

Susceptible Single Nucleotide Polymorphisms in Exon 10 and Intron 9 of Complement Factor H Gene in Patients With Age-related Macular Degeneration



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ABSTRACT

Background: Age-related macular degeneration (AMD) is the leading cause of vision loss in the elderly. Although it has been shown that Y402H polymorphism in the CFH gene was strongly associated with AMD in the Iranian population, there were no data on other single nucleotide polymorphisms (SNPs), which have the most significant association with AMD. This study aimed to investigate hot point regions in exon 10 and intron 9.

Materials and Methods: One hundred and sixty-six AMD patients and 69 controls were recruited. Their blood was collected in the tubes containing EDTA. Then, DNA was extracted from the blood, and its quality was evaluated. Primers were designed for intron 9 and exon10 sequencing. A viral polymorphisms analysis software named CEQ was used for the analysis of putative polymorphisms.

Results: We noticed three polymorphisms in study cases: rs7535263 and C66379A in intron 9 and rs2274700 in exon 10. Based on the McNamara's test (rs7535263 and rs2274700) and the Phi and Cramer's test (C66379A), a significant difference was found between the control and patient groups regarding rs7535263 and rs2274700 polymorphisms.

Conclusion: We found a synonymous or silent mutation, A473A, rs2274700 in exon 10 in 85% of patients. From two intronic SNPs, just rs7535263 showed association with the disease in studied patients living in Gilan Province, Iran. Although no significant relationship was found between controls and patients regarding the C66379A allele, it would be important that no other sources have reported C66379A polymorphism in AMD yet.

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1. Introduction

Age-related macular degeneration (AMD) is the main cause of blindness in adults older than 55 years in industrialized countries [1]. The disease is divided into two types: dry or nonexudative (90%), which is characterized by drusen, hyperpigmentation or depigmentation, and geographical atrophy in the macula and the wet type or neovascular AMD (10%) [2, 3]. The most common findings of AMD pathophysiology are retinal pigment epithelium (RPE) incapability in phagocytosis and metabolism, development of sub-RPE depositions called drusen which induces a gap between the RPE cells and the underlying Bruch's membrane, complement Factor H chemical integrity, which seems to play an essential role in AMD, neovascularization of the choroid that is due to the overexpression of angiogenic growth factors [4, 5].

Three major risk factors for AMD are age, smoking, and inheritance. There is a greater risk of disease in the white population than in blacks and Hispanics [6]. Because of the presence of different phenotypes, genetic studies cannot strongly refer to a single gene, even if all different stages of the disease be included in the study [7].

Studies have identified the most functionally related genes on the chromosomes 31-25 q1 and 26 q10 of the human genome [8-11]. Complement factor H (CFH) controls the activated complement cascade [12]. Accumulation of the components of the complement system, such as the C5b-9 complex, has been observed in the Bruch's membrane and in the drusen bodies, which may be the result of disruption in inhibition of complement cascade [13].

It has been shown that some variants of the CFH gene increase the risk of developing AMD. Significant relationships between the different variants of CFH and AMD in many populations [14] have been demonstrated. Nonyane BAS et al.'s results suggested a positive association between the prevalence of late AMD and Y402H risk-allele frequency across ethnicities, except in those of African descent [15].

Some CFH variants revealed a significant correlation to the response against prescribed anti-angiogenic drugs for AMD [16]. Haplotypes that do not encode the Y402H variant reveal a strong connection with the vulnerability to AMD [17].

Genome-wide screening studies have been conducted to link the nominated polymorphisms and AMD. Among

the CFH variants, two of them are strongly associated with increasing AMD risk, I62V (rs800292 in exon 2) and Y402H (rs1061170 SNP in exon 9) [18]. Y402H-encoding variant (rs1061170) shows prevalence for any type of AMD. The highest occurrence rate for this allele has been found in advanced AMD and the least in non-affected individuals.

The homozygous form of the Y402H variant in the CFH gene increases AMD risk by a factor of about 7.4. This change happens in the CFH binding region to heparin and C-reactive protein [19]. Nazari Khanamiri et al. showed that Y402H polymorphism was strongly associated with AMD in the Iranian population [20]. Francis et al. showed an association between 7 types of Single nucleotide polymorphisms (SNPs) in the CFH gene and AMD, including the strongest association with A473A (rs2274700) in exon 10: a silent mutation [21].

Scholl et al. showed an association between rs2274700 in exon 10 and SNPs, rs1410996 (intron 14), and rs7535263 (intron 9) in their study on AMD patients [22].

According to similar studies, inside the CFH gene, an SNP group containing three polymorphisms—a synonymous SNP in exon 10 (rs2274700) and two intronic SNPs (rs1410996 and rs7535263)—represent a vivid association with disease susceptibility compared to Y402H variant [17, 21]. They are variants that seem to be important in regulating the expression of CFH, or other relative complement genes [17, 23].

Out of 20 SNPs that had shown the most significant association with AMD (even more than Y402H), 9 SNPs reside in intron 9 [17]. On the other hand, there were no data on the SNPs described above in the Iranian population. So, we aimed to investigate whether variants, including rs2274700 and rs7535263, of the CFH gene, were associated with AMD in Gilan Province, northern Iran, versus sex- and age-matched healthy subjects from the same area.

2. Materials and Methods

Selection and sampling of the patients

This study was approved by the Ethics Committee of the Ophthalmic Research Center, Shahid Beheshti University of Medical Sciences (IR.SBMU.ORG.REC.1394.014), and was compatible with the Helsinki Declaration. Informed consent was obtained from all participants. A total of 166 patients with AMD and 69 con-

controls were included in this study. The AMD severity was classified into early, intermediate, and advanced stages.

Study patients

This study was part of a population-based study on the prevalence of AMD in Gilan Province that was performed on all individuals over 50 years of age with a cluster random sampling method from different urban and rural areas in Northern Iran [24]. The questionnaires regarding the epidemiological data, economic status and occupation, and exposure to sunlight were filled. Then general examinations, including taking the history of diseases such as systemic hypertension, hyperlipidemia, and diabetes, were performed. In the next step, blood pressure and visual acuity were recorded, and then complete ophthalmologic examinations, including external and slit lamp examinations and intraocular pressure measurements, were performed. Later, funduscopy with dilated pupils focusing on the signs of AMD was accomplished. The presence of pigmentary changes, subretinal depositions and or fluid, pigment epithelial detachment, subretinal bleeding, and scar and geographic atrophy were assessed. Finally, color fundus photography for both eyes of each participant was done.

The inclusion criteria were as follows. First, patients were included with no or low media opacity in both eyes and good quality of color fundus photographs for the second opinion of board-certified retina subspecialist of the study group for AMD severity. Second, patients were included who had definite AMD diagnoses in at least one eye based on a simplified severity scale for AMD. The definition is as follows: early AMD with medium-sized drusen $>63 \mu\text{m}$ and $\leq 125 \mu\text{m}$ and no pigmentary abnormalities, intermediate AMD as the presence of at least one large drusen ($>125 \mu\text{m}$) with or without pigmentary changes or medium-sized drusen with pigmentary changes, and advanced AMD as center involved geographic atrophy or choroidal neovascularization. The purpose is to minimize the chance of inclusion of patients with a very low risk of AMD progression. Third, enough blood sample size for DNA extraction in addition to informed consent for blood DNA analysis.

Controls were the 50+ year people of Guilan Eye Study (GES), study without any symptoms and signs of AMD and no retinal disease which may affect the vision. We included patients with unremarkable funduscopy exams, which were reconfirmed by one of our board-certified retinal subspecialists from color fundus photography. Table 1 presents the patients' characteristics.

Study samples

After selecting the patients, 10 mL of their blood was collected in the tubes containing EDTA. DNA was extracted, and the DNA quality was evaluated. Primers were designed for intron 9 and exon 10 for sequencing. Later, we searched for SNPs and performed the statistical analysis.

DNA extraction

DNA of all samples was extracted using a QIAamp DNA Mini Kit. In summary, 200 μL of blood sample was mixed with 200 μL of the lysing buffer and 20 μL of proteinase K enzyme and was incubated at 56°C for 20 minutes. Then, 200 μL of absolute ethanol was added to each sample and was mixed vigorously. Next, it was washed two times with buffers, and ultimately the eluted solution was stored at -20°C.

DNA quality control

Two different methods were used to analyze the extracted DNA quality. Spectroscopy examination was performed at the wavelengths of 260, 280, and 230 nm. Absorption ratios of 260/280 and 260/230 of about 1.8-2 were considered for the inputted samples. The DNA concentration in the downstream manipulations was optimized to 40-50 ng for each reaction. Human beta-actin primers that amplify DNA fragments of about 500 bp were used to determine the relative quality of DNA and the presence of inhibitors in amplification procedures.

Primer design

The human CFH gene FASTA file was recruited from the gene bank (http://www.ncbi.nlm.nih.gov/nucleotide/NG_007259.1). Using the Mfold software, the secondary regions related to exon and intron were identified. The parameters of OLIGO 6 software were determined according to the ΔG of Mfold. The accuracy and the quality of the primers were again checked in the Beacon Designer software 8 and the Allele ID 7 and finally controlled by BLASTn and the primer blast. Table 2 presents the primers used to amplify the desired fragment.

PCR reaction

The steps of the PCR reaction were as follows. PCR reaction was performed using a primer pair specifically to amplify the region of interest, including exon 10 and an adjacent element of intron 9 encompassing 500 nucleotides (total amplicon length; 902 bp) with AmpliTaq Gold PCR Master Mix (Thermo Fisher Scientific,

Brussels, Belgium). We applied a total reaction volume of 10 uL under optimized PCR conditions (94°C for 10 min followed by 40 cycles of 95°C for 40 s, 63°C for 40 s and 72°C for 50 s, followed by an incubation step at 72°C for 5 min). The PCR was carried out using MyCycler™ Thermal Cycler-Bio-Rad. PCR products were run through 1.5% agarose gel and visualized by using UV transillumination.

DNA sequencing

The amplicons were subjected to direct gene sequencing (Arya Taq gene Azma, Eurofin Genomics, Bayern, Germany). The sequences were aligned and compared with reference sequences using GenBank BLAST, a basic local alignment search tool.

After designing the primer and performing a PCR experiment, the method recruited to analyze the results is essential. Based on the experiences in viral polymorphisms analysis, in our group, CEQ-8000 software was used to include the convenient features to analyze the sequencing data. The most important feature of this package is simultaneous analysis of the reverse and forward data and aligning the sequences with the reference gene. Another feature of this software is to identify known mutations. So, it reports the final results as known and unknown polymorphisms.

The CEQ software can report the final sequencing results as FASTA. Accordingly, using the CluſtalW2 software, pairwise alignment analysis determined the results of sequencing with the refSeqGene, and specific polymorphic changes were identified in the reference gene. In the next step, using the SPSS, the presence and absence of the significant difference between the control and patient groups were examined.

3. Results

From patients in the GES study, we included 166 AMD patients based on inclusion criteria as defined in methods. Three polymorphisms in cases as follows, rs7535263, C66379A in intron 9, and rs2274700 in Exon 10 were found. Figure 1 shows intron 9 and the location of the mutant nucleotides inside the intron 9 (rs7535263 and C66379A). Figure 2 displays exon 10 and the location of the mutant nucleotide inside the exon 10 (rs2274700). SnapGene®3.2.1 designed both figures. According to Figure 3, the frequencies of mutation in the rs7535263 and C66379A were seen in 19% and 60% of the patients, respectively. In addition, the mutation in the rs2274700 in exon 10 was observed in 85% of patients. The frequencies of these mutations in the control group were 46%, 52%, and 40%, respectively.

The polymorphisms of the rs7535263 and rs2274700 in controls and patients were analyzed by SPSS v. 23 and

Table 1. Characteristics of AMD patients included in genetic analysis and control patients.

Female	OD				OS				
	Early	Intermediate	Advanced	No AMD	Early	Intermediate	Advanced	No AMD	
AMD Patients; Total, 166	81 48.7%	92 55.4%	40 24%	10 6%	24 14.4%	92 55.4%	41 24.6%	12 7.2%	21 12.6%
Controls; Total, 69	31 45%	N/A		69 100%	N/A		69 100%		

N/A: Not applicable.



Figure 1. Intron 9 and the location of rs7535263 and C66379A



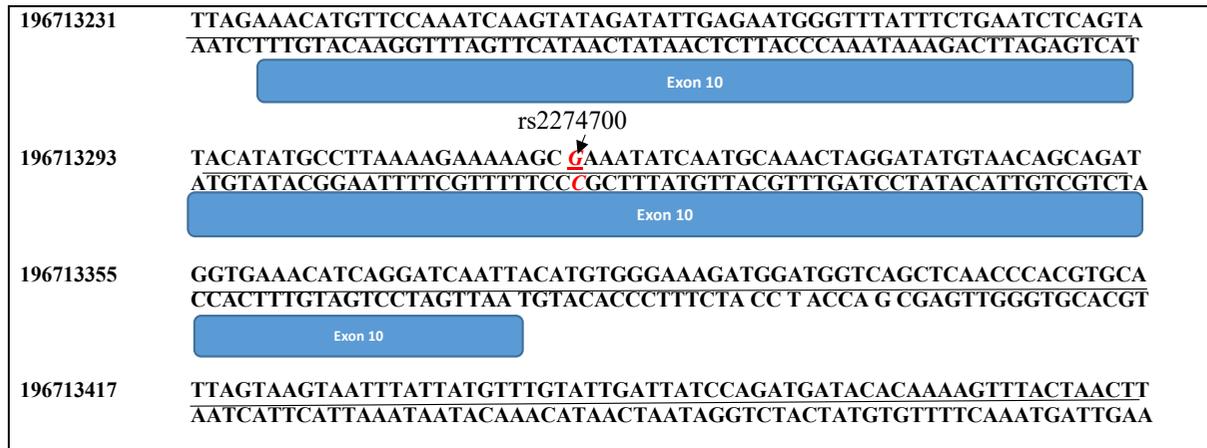


Figure 2. Exon 10 and the location of rs2274700

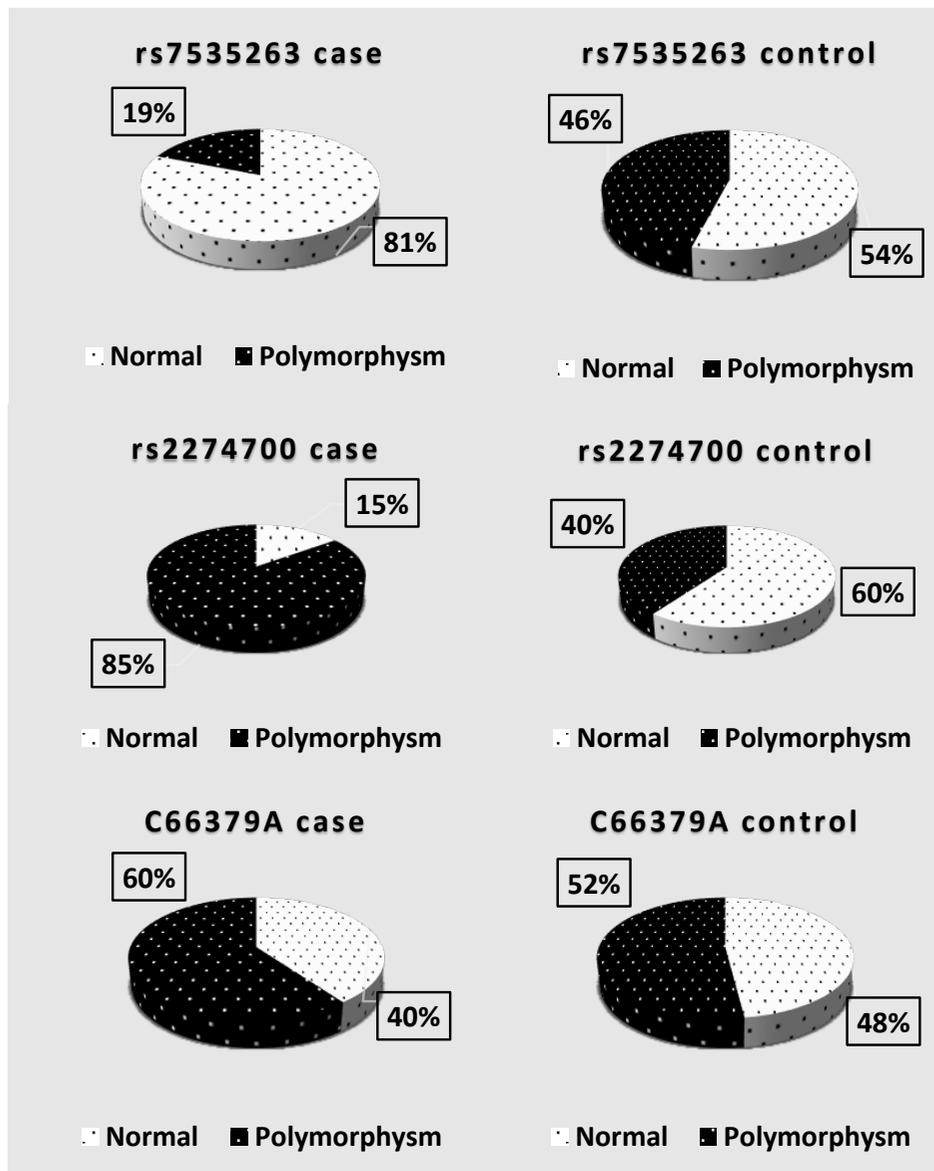


Figure 3. The frequency of detected SNPs in cases and controls



Table 2. Primers used to amplify the desired fragment

Primer	Sequence (5'→3')	Length	Tm	GC%	E-value	Product Size
Forward	CCTCGTCTTCTTGAAGTCCACA	23 mer	66.5	48	3e-04	902 bp
Reverse	AGCCTGAATGGAAAGACAGACT	22 mer	65.7	46	0.001	

	Hairpin Delta G Kcal/mol	Self-Dimer Delta G Kcal/mol	Cross Dimer Delta G Kcal/mol
Forward	-0.6	-0.6	-3.6
Reverse	-1.2	-1.2	



Excel 10 software. The results of the significant level of the above tests were presented in Table 3.

As shown in Table 3, there was a significant difference between the control group and the patients for the rs7535263 allele ($P < 0.05$). In addition, in the rs2274700 allele, the P value between the control group and the patients was 0.001 (less than 5%). Therefore, there was a significant difference between the control and patient groups for rs7535263 and rs2274700 alleles. Regarding the C66379A allele, the significance level of Phi and Cramer's V test was greater than 0.05, and therefore the hypothesis, which indicates the lack of relation between the two variables, was not rejected. Thus, there was no significant relationship between controls and the patients regarding the C66379A allele.

4. Discussion

We found three polymorphic sites, nominated as rs2274700 (exon 10), rs7535263, and C66379A (intron 9) alleles. The McNamara's test applied to rs7535263 and rs2274700 to examine the presence or absence of corre-

lation between the control, and patient groups showed a significant difference between the control and patient groups for rs7535263 and rs2274700 polymorphisms. However, Phi and Cramer's V test could not show such a significant correlation for C66379A in intron 9.

In 2007, Francis et al. [21] showed a strong association between CFH polymorphism and the advanced stage of AMD in three independent Caucasian populations. They found 7 types of SNPs in CFH gene that were significantly associated with AMD: rs529825, rs3766404, rs1061147, rs800292, rs1061170, rs203674, and rs2274700. Their results were highly similar to the original published articles. Francis et al. found the strongest association between AMD and A473A (rs2274700) in exon 10. We also found rs2274700 in Exon 10. The mutation inside the exon 10 at position 66808 (codon 473: GCG>GCA) not leading to an amino acid switching. Both GCG and GCA are codons for alanine amino acid. This kind of single nucleotide polymorphism was named synonymous or silent mutation. In the current study, rs2274700 was observed in 85% of patients,

Table 3. Significance level of statistical tests

Data analysis	Groups	McNemar's test	Cramer's V test	Phi test
		Significance		
rs7535263 Control		0.036	-	-
rs7535263 Case				
rs2274700 Control		0.001	-	-
rs2274700 Case				
C66379A Control		-	0.960	0.960
C66379A Case				



which revealed a significant difference between the control and patient groups.

Li et al. (2006) [17] examined the polymorphism in and around the CFH gene in 726 affected individuals and found that 20 polymorphisms showed a stronger association with the disease when compared to the Y402H variant. They determined a series of 4 common haplotypes, two of which were related to the incidence of the disease, and the other two were protective against the disease. The strongest association was still for the SNP, synonymous with the rs2274700 variant (A473A).

Out of two intronic (intron 9) SNPs (rs7535263 and C66379A), only rs7535263 showed association with the disease. Scholl et al. (2007) [22] showed a strong association between three SNPs; a synonymous SNP in exon 10, rs2274700, and two intronic SNPs, rs1410996, and rs7535263 in their study on AMD patients. We also found rs7535263 SNP and intronic synonymous rs2274700 (A473A) in studied patients in Gilan Province, Iran.

Concerning C66379A (intronic SNP) allele, the null hypothesis, which indicates the lack of relation between the two variables, was not rejected. So, no significant relationship between controls and patients for the C66379A allele was found. No other sources have reported C66379A polymorphism in AMD yet.

These two SNPs lie in intron 9 of the gene (GenBank accession NM_000186) for complement factor H (CFH), located on chromosome 1q31. Both of them are noncoding, and neither alter a conserved sequence. It would be interesting to explore these SNPs in linkage disequilibrium with functional SNPs throughout chromosome 1q.

In this study, DNA samples came from a large population, based on a study designed as cross-sectional random cluster sampling from rural and urban areas in Northern Iran [24]. After a complete ocular exam, we included 166 patients with at least one eye having more than one medium-sized drusen or one large drusen with or without pigmentary changes or more severity. The strong side of our study is that the control group was randomly matched by age and sex from non-AMD patients of the same study and same ethnicity. One criticism of case-control association studies is that the population arrangements can result in false-positive results, which might turn to a minimum with cluster random population-based study designs as ours. If the cases and controls were from different ancestry, with different allele frequencies, we might detect the population differences instead of SNPs associated with

the disease. The finding of just two SNPs implied the absence of genetic stratification. The significance of the tests was not escalated; therefore, these two associated SNPs (rs7535263 and rs2274700) seem to be significantly associated with the disease.

The limitations of our study are the low access to blood samples and the inclusion of patients with early AMD. It might be preferred to include patients with both eyes diagnosed as AMD and at least one eye intermediate AMD grade. Another important limitation is that the study was conducted in only one province with possible ethnic differences from other Iran provinces. However, the people from other parts of the country have emigrated to this province because of the desirable weather and good socioeconomic conditions of Gilan Province, since many years ago, and has the highest rate of population for the area.

According to similar studies, an SNP group contains a synonymous SNP in exon 10 (rs2274700) and two intronic SNPs (rs1410996 and rs7535263). They represent a strong association with disease susceptibility compared to the Tyr402His variant [17, 21]. These polymorphisms make a CFH haplotype along with the His402 variant. They seem to be important in regulating the expression of CFH or other relative complement genes [17, 23].

Regarding the results of statistical analysis with a 95% confidence level, the continuation of the study with more clinical sampling in terms of odds ratio and the confirmation of the clinical relationship between polymorphisms and severity of illness seems necessary.

Plotkin et al., in an article published in Nature in 2011, stated that mutations, in contrast to their names, have effective results on cellular processes in all species [25]. The prevailing conventional belief in molecular biology states that mutations that do not change the code of amino acids do not affect the protein sequence and, consequently, cellular function, the fitness of an organism, and evolution [26, 27]. In most sequenced genomes, codons of the same amino acid are not used in the same sequences. This phenomenon is called the codon usage bias in the term. Coding bias happens inclusively and can be very strong. Some species, like *Thermus thermophilus*, almost do not have some codons [25].

Two categories of evidence prove that synonymous mutations can have functional consequences. The first evidence was based on findings in which there was a codon usage bias in many organisms, indicating that even codons of the same meaning were under evolutionary

pressure. The latter was based on the fact that progress in understanding the production and folding of the protein led to a discovery, which provided a framework for the mechanism and concept to understand this phenomenon. Synonymous mutations can cause inappropriate mRNA splicing and a variety of human diseases. Further evidence suggests that synonymous single nucleotide polymorphisms (SNPs) can affect protein stability and, consequently, expression and enzymatic activity of the protein and have functional and clinical effects. In general, mutations also affect the phenotype through the effect on splicing and translation accuracy, mRNA structure, and spatial shape of the protein. Examples of diseases associated with synonymous mutation include Treacher-Collins syndrome, cystic fibrosis, and Seckel syndrome. There is also an SNP in the Wilms tumor 1 patient with acute myeloid leukemia that correlates with disease progression. In addition, a single nucleotide polymorphism in the heat shock protein B7 family of cardiomyopathy is associated with vulnerability. The synonymous mutation in ABCB7 is associated with protein folding and its specificity to the substrate and therapeutic results [26, 27].

5. Conclusion

In conclusion, due to the displacements of the nucleotides mentioned above and the changes in polarity and hydrophobicity, a bioinformatics study on the structure and function of modified forms of proteins compatible with polymorphic genes is necessary.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Ethics Committee of the Ophthalmic Research Center, Shahid Beheshti University of Medical Sciences (Code: IR.SBMU.ORB.REC.1394.014)

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Authors' contributions

Conceptualization and supervision: Zahra-Soheila Soheili, Hamid Ahmadi, Siamak Moradian; Methodology: Samira Kheitan, Shamila D. Alipoor, Abouzar Bagheri, and Seyed Ghader Azizi; Investigation, writing

– original draft, and writing – review & editing: Zahra-Soheila Soheili, Narsis Daftarian; Data collection: Shahram Samiee; Data analysis: Shahram Samiee; Funding acquisition and resources: Zahra-Soheila Soheili, Hamid Ahmadi, Shahram Samiee.

Conflict of interest

The authors declared no conflict of interest.

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