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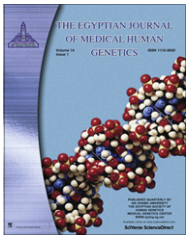

ORIGINAL ARTICLE

Clinical characteristics and analysis of *HFE* gene variants (C282Y and H63D) in Jordanian Arab patients with age-related macular degeneration

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Abstract Age-related macular degeneration (AMD) is a complex genetic disorder with multiple etiologies. Multiple genes as well as environmental effects are thought to play a role in causing AMD. Recent evidence pointed that elevated iron overload, resulting from hereditary defects of iron homeostasis, is associated with retinal degeneration and consequently plays a role in the pathogenesis of AMD. Hemochromatosis is a genetic disorder in which excess iron is absorbed from the diet and deposited in different tissues, primarily caused by mutations in *HFE* gene. Two major mutations in *HFE* are responsible for most hemochromatosis cases, namely, C282Y and H63D.

In this work we gathered information relating to 37 AMD patients from Jordan, and investigated the potential association between hemochromatosis, or more specifically, carrier state for a mutation in *HFE* gene (which may moderately increase dietary iron absorption) and AMD, given the effect of elevated iron levels on AMD occurrence. Questionnaires and blood samples were collected from patients visiting the eye care clinic in the King Abdullah hospital in Jordan. DNA was extracted from patient samples and mutations in *HFE* were genotyped (using polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP), and DNA sequencing) and compared to 106 control samples.

We could not detect C282Y (rs1800562) variant in our patient population or in the controls. For carrier status with H63D (rs1799945) we had 30.3% compared to a frequency of 22.7% in the

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controls ($p = 0.37$). H63D allele frequency was 15.2% in our patients compared to 11.8% in the controls ($p = 0.30$).

H63D variant seems to be more frequent in AMD patients though not reaching a significance of $p = 0.05$. To date, this is the first attempt to link *HFE* (particularly, H63D) mutation to AMD.

1. Introduction

Retinal degeneration has been suggested recently to be promoted by elevated iron levels [1]. Hereditary defects in iron homeostasis that results in elevated retinal iron levels are suggested to associate with retinal degeneration. These hereditary defects include hemochromatosis, aceruloplasminemia, and pantothenate kinase associated neurodegeneration (PKAN) [2]. Age-related macular degeneration (AMD) has recently been suggested to be caused (at least in some cases) by elevated iron levels in the retina (which might be caused by one of the hereditary defects of iron homeostasis) that leads to retinal degeneration and, consequently, to AMD [2,3]. Suggested involvement of iron in AMD by at least three lines of evidence; elevated iron levels in retinas of AMD patients [4], development of signs of macular degeneration in iron-overloaded retinas of ceruloplasmin/hephaestin and hepcidin knockout mice [5,6], and the development of early-onset drusen in an aceruloplasminemia patient with retinal iron overload [7].

The prevalence and mechanisms of iron overload in AMD are not known. One probable cause of iron overload is a mutation in *HFE* (the hereditary hemochromatosis gene). Penetrance of Hemochromatosis is relatively low, not every one with *HFE* mutation would develop hemochromatosis, but most often carriers of *HFE* mutations would result in a more than normal absorption and consequent deposition of iron in body tissues including the retina. Carriers of C282Y mutation are high in the population and it reaches up to 10%. Here, we describe a set of Jordanian Arab AMD patients and investigate the frequency of C282Y and H63D variants in patients and controls to examine whether *HFE* mutations play a role in increasing the risk of AMD.

2. Subjects and methods

2.1. Subjects

A total of 143 individuals have participated in this study. Thirty-seven patients with Age-related Macular Degeneration (AMD) were recruited from the eye clinic in the King Abdullah University Hospital in Irbid, Jordan. Average age of patients was 71 years. Thirty percent (11/37) of patients were females. Disease was diagnosed by one of the authors (S.A.) using the AREDS staging system [8]. Optical coherence tomography (OCT) and/or Fundus Fluorescein Angiography (FFA) were done to confirm diagnosis to all patients with active disease. No investigations were done to end stage wet AMD with macular scar or to patients with dry AMD with small size drusens and very good visual acuity with no suspicion of sub-retinal fluid. Clinical and demographic data were recorded for each patient. Controls were the attendants of the hospital with no signs of eye problems or hemochromatosis. Positive control samples with homozygous mutant and heterozygous mutants were kindly provided by Dr. Osama Smadi (King Faisal hospital, Saudi Arabia) and Dr. Richard Allen (University of Oklahoma Health Sciences Center, USA). An informed consent was obtained from all participating individuals. This study was approved by the Institutional Ethics Review Board of the Jordan University of Science and Technology.

2.2. Genotyping

Peripheral blood samples were collected from all participants. DNA was extracted using DNA purification Kits (Qiagen). Manufacturer's protocol was followed. Two amplicons were amplified by PCR for genotyping the three variants [9]. Primers were designed using primer3 software [10]. Sequences for primers used to genotype C282Y were: forward 5′-AAGGATAAGCAGCCAATGGA-3′ and reverse 5′-CCATAATTACCTCCTCAGGCACT-3′ (GenBank Accession number NT 007592.15). To genotype H63D primer set used was: forward 5′-GTTTGAAGCTTTGGGCTACG-3′ and reverse 5′-TACCCTTGCTGTGGTTGTGA-3′ (GenBank Accession number NT 007592.15). Amplifications were performed in a total volume of 25 μl using the thermal cycler (icycler, Bio-Rad). Each PCR reaction contained 12.5 μl GoTaq Green Master mix, 2× (dNTPs, $MgCl_2$, PCR buffer and Taq polymerase) (Promega), 2.5 μl of each primer (1 μM final concentration) (Alpha DNA), 4.5 μl nuclease free water, and 3.0 μl (75 ng) of DNA template. After an initial step of 5 min at 95 °C, the samples were processed through 35 temperature cycles of 30 s at 94 °C, 30 s at 56 °C, and 30 s at 72 °C, and a final extension step of 72 °C for 10 min. PCR products were run on 2% agarose gel and visualized by UV light after ethidium bromide staining to assess the correct sizing of the amplified product. Following PCR, products were digested by *RsaI* for C282Y and *MboI* for H63D (Fermentas). The restriction fragments of PCR products were separated by electrophoresis on a 3% agarose gel containing 10 μg/ml ethidium bromide, and visualized by UV light and genotypes recorded. Positive and negative controls were included in each run. Direct sequencing of PCR-amplified fragments was performed to confirm the RFLP detected variants. Sequencing was performed in both directions (forward and reverse). The purification of PCR product and sequencing reaction were done by Macrogen Company (Korea). The sequences were analyzed using Bioedit software available online (http://www.mbio.ncsu.edu/bioedit/bioedit.html).

2.3. Statistical analysis

Data were entered in excel for the calculation of allele and genotype frequencies. Hardy–Weinberg equilibrium (HWE) was tested to determine if the population was fulfilling the HWE at each mutation locus. It was assessed in the observed genotype distribution with a Chi square test. Allelic association *p*-values were determined using a Chi square test in cases

versus controls. Genotypic association *p*-values were determined by the Freeman-Halton extension of Fisher's exact test for a 2×3 contingency table which evaluates the occurrence of all three genotypes as an array between the cases and controls [11]. A web-based calculator was used to compute *p*-values (Vassar Stats). A p value < 0.05 was considered to be statistically significant for both tests. Using the Genetic Power Calculator (http://pngu.mgh.harvard.edu/~purcell/gpc/cc2.html) we found that we need **31** cases to get 80% power to detect allelic association for H63D (assuming a relative risk of two for Aa and six for AA genotypes, alpha = 0.1). Number of cases needed to get 80% differs by the marker because markers have different frequencies. C282Y was not present in our population (patients and controls). Consequently, our sample size is enough given the allele frequency of the marker.

3. Results

We had 37 AMD patients and 106 unaffected controls. Complete clinical data were obtained for all AMD patients and complete demographics were obtained from all participating individuals. Genotyping was done for 33 patients (4 patients did not provide blood samples) and all controls. Among AMD patients 11 were females (30%) and 26 were males while among controls there were 57 females (53%) and 49 males. For patients, mean age was 71.6 ± 6.9 years, while for controls mean age was 62.8 ± 7.9 years. Average age of diagnosis for patients was 68.5 years. Dry AMD constituted 42% of patients, wet AMD were 25%, and dry and wet AMD were 33%. Only 4 patients (11%) had a single eye involvement, and the rest had bilateral AMD. Twelve AMD patients were diabetic (32%). Twenty-six patients (70%) were receiving Bevacizumab for treatment and 11 (30%) were not receiving treatment.

We genotyped patient and control samples using PCR of the region continuing the variants followed by restriction enzyme digestion of the amplified product. Band patterns were scored and samples genotyped (Fig. 1). We sequenced a subset of data to confirm our RFLP results (Fig. 2). We could not detect C282Y variant in our patients or in the controls. Genotypic and allelic frequencies of patients and controls for H63D are summarized in Tables 1 and 2, respectively. Genotypic frequencies met HWE expectations. We could not detect association between H63D genotypes ($p = 0.37$) and alleles ($p = 0.30$) with AMD phenotype.

4. Discussion

This is the first paper to discuss AMD disease in Jordan, and the first, to the best of our knowledge, to assess the contribution of H63D variant in *HFE* gene to AMD. Iron is an important cofactor in multiple proteins and enzymes that are involved in cellular metabolic functions including DNA replication, membrane biogenesis, oxygen transport, and respiratory chain electron transfer. AMD patients have iron metabolism abnormalities which may aggravate oxidative retinal injury [12]. While the probability that iron accumulation is simply a result of as opposed to a cause to AMD might not be an invalid idea, much evidence points to a more or less direct role in AMD pathogenesis.

H63D has an established role in iron homeostasis [13]. H63D also affects transferrin saturation levels [14], and heterozyotes for H63D showed a significant increase in transferrin saturation and serum ferritin levels as compared to *HFE* wild type controls [15–17]. *HFE* H63D mutation frequency shows high variability worldwide [18]. It ranges from 23.0% in Bulgaria [19] to 3.5% in Ecuador [20]. Allelic frequency of H63D in Jordan is 11.25% [18].

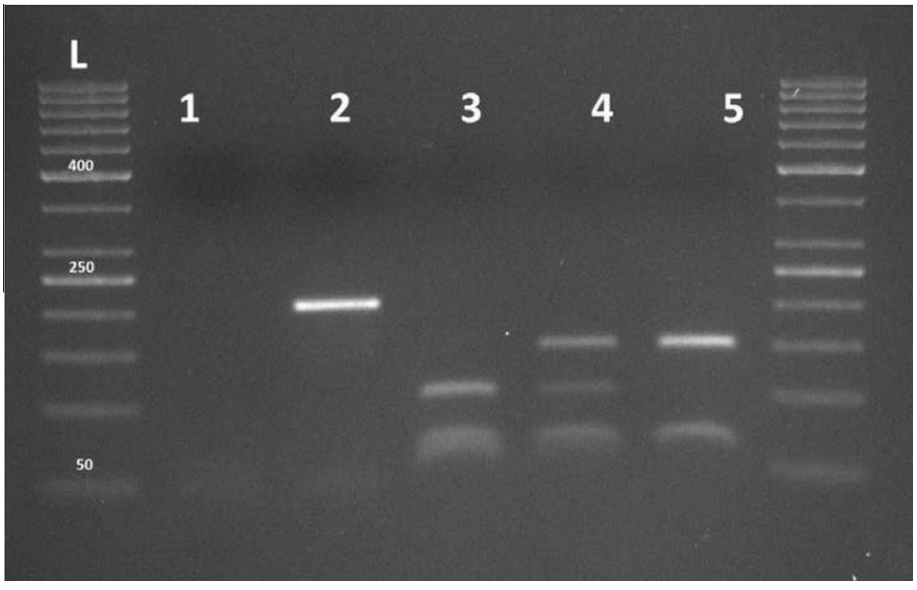

Figure 1 Gel electrophoresis for *HFE SNP* (*H63D*) PCR product and its RFLP products. Lane L represents 50 bp DNA ladder, Lane 1 negative control, Lane 2 (202 bp) PCR product, Lane 3 (99 + 59 + 48 bp) represents RFLP product for homozygous GG, Lane 4 (147 + 99 + 59 + 48 bp) represents RFLP product for heterozygous CG, Lane 5 (147 + 59 bp) represents RFLP product for homozygous CC.

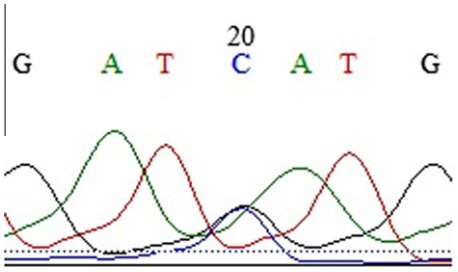

Figure 2 Sequencing electropherogram of *HFE SNP* (*H63D*), vertical arrow points to the rs1799945 polymorphism.

Table 1 Genotype distributions of *HFE* H63D variant in AMD patients and controls.

Genotype	AMD patients (%)	Controls (%)	*p*-value[a]
GG	23 (69.7)	83 (78.3)	0.37
GC	10 (30.3)	21 (19.8)	
CC	0 (0)	2 (1.9)	
Total	33	106	

[a] Genotypic association *p*-values were calculated using an extension of Fisher's exact test for a 2×3 contingency table.

In our study we could not detect any difference in the distribution of H63D alleles in AMD patients as compared to controls, and thus, H63D mutation does not appear to play a major role in increasing the risk of AMD. Consequently, the potential involvement of iron in modulating oxidative injury in AMD might be not related to different functions of H63D and wild type alleles.

Various factors could be taken into account with respect to these results. H63D is less penetrant than C282Y mutation and thus an effect of C282Y on increasing the risk of AMD is possible. C282Y is so rare in the Jordanian population; we detected none in our patients or in our controls. In a recent study Asleh et al. could not find association between AMD and C282Y due to the low frequency of the allele in the same region [21]. It is possible that H63D alleles increase the risk of AMD in a small number of cases and its effect is minor, thus such an effect might not be detectable in this study. The small number of patient samples might also contribute to a lower power to detect this minor effect. Finally, environmental factors might play a role in exacerbating or modulating the effect of H63D mutation on AMD risk, such potential factors were not analyzed in this study.

In conclusion, we analyzed the association of two hemochromatosis *HFE* mutations; C282Y, and H63D in AMD patients. C282Y, the major hemochromatosis causing mutation was completely absent from our AMD patients and controls. This is consistent with the fact that hemochromatosis has a very low prevalence in Jordan and that it is not merely under-diagnosed. H63D mutation was found to be common with 11.7% allele frequency, but it plays a minor role in hemochromatosis as compared to C282Y mutation. We analyzed the association of H63D mutation with the risk of developing AMD but we could not detect such association in our test group suggesting that this mutation does not play a major role in increasing the risk of developing AMD in Jordanian Arab population.

Brief summary statement

We describe the clinical characteristics of a sample of AMD patients from Jordan and assess the contribution of HFE gene variants C282Y and H63D in increasing the risk of AMD by an association analysis.

Acknowledgments

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Table 2 Allele distributions of *HFE* H63D variant in AMD patients and controls.

Allele	AMD patients (%)	Controls (%)	*p*-value[a]	Odds ratio (95% CI)
G	56 (84.8)	187 (88.2)	0.30	1.34 (0.61–2.95)
C	10 (15.2)	25 (11.8)		
Total	66	212		

[a] Allelic association *p*-values were calculated using a Chi square test.

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