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Frequency of the hemochromatosis gene (*HFE*) variants in a Jordanian Arab population and in diabetics from the same region

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Abstract. Hereditary *HFE*-linked hemochromatosis is a frequent recessive disorder among individuals of northern European ancestry. The clinical characteristic of this disease is the gradual accumulation of iron in internal organs, which ultimately may lead to organ damage and death. Three allelic variants of *HFE* gene have been correlated with hereditary hemochromatosis: C282Y is significantly associated with hereditary hemochromatosis in populations of Celtic origin, H63D and S65C are associated with milder form of iron overload. In this study we performed mutation analysis to identify allele frequency of the three variants of *HFE* gene in Jordanian Arab population, to assess deviations of these frequencies from those detected elsewhere, and to determine if there is an increased frequency of these variants in a diabetic population (Type 2 diabetes) from the same area. DNA was extracted from blood samples of 440 individuals attending King Abdullah University Hospital for ambulatory services. We used polymerase chain reaction (PCR) to amplify exons 2 and 4 of the *HFE* gene then restriction fragment length polymorphism (RFLP) method to detect the variants. There were neither homozygous nor heterozygous for C282Y variant. For the H63D variant, 0.68% were homozygous and 21.1% were heterozygous. For the S65C variant, there were no homozygous and 0.23% were heterozygous. Allelic frequencies were, 0%, 11.25%, and 0.11% for C282Y, H63D, and S65C, respectively. Our samples were subdivided into two categories of type 2 diabetic (89 cases) and controls (blood donors, 204 cases) and compared with regard to the H63D variant. Both groups did not have homozygous H63D variant. H63D heterozygous in diabetics were 23.60% and in blood donor controls 22.55%. Allelic frequency of the mutant H63D allele was 11.80% in diabetics and 11.27% for the blood donor controls. This is the first study to show the frequency of the three hemochromatosis gene variants in Jordan with the interesting finding of no C282Y allele detected in 440 samples. Additionally, no significant difference was observed in H63D variant frequency in type 2 diabetics as compared to controls.

Keywords: Hemochromatosis, *HFE*, diabetes, H63D

1. Introduction

Hereditary *HFE*-linked hemochromatosis (OMIM 235200) is an autosomal recessive disorder of iron metabolism. It is caused by an increased absorption of iron from the diet and consequent deposition of the extra iron in parenchymal cells of the liver, pancreas and heart. This deposition leads to abnormality in organ functions and results in multiple manifestations including primarily; liver cirrhosis, diabetes mellitus, hyperpigmentation of skin, and cardiac failure (reviewed in 1). Phenotypic expression usually starts with fatigue followed by symptomatic organ involvement of liver, heart, endocrine system and joint disease. Hemochromatosis gene, *HFE*, was discovered in 1996, with homozygosity for C282Y (c.845G $>$ A, rs1800562) variant being most commonly associated with the disease [2]. C282Y seem to have originat-

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ed in north Europe around 2000 years ago and spread since then [3]. The frequency of this variant ranges from 14% in Ireland [4] to 0% in different countries [5]. Two other variants in *HFE* are thought to increase the risk of hemochromatosis namely; H63D (c.187C > G, rs1799945) and S65C (c.193A > T, rs1800730). Hemochromatosis has a low penetrance [6]. It has an age of onset in midlife, being later in females than males.

Diabetes mellitus is the most commonly associated endocrine abnormality with hemochromatosis. Not all hemochromatosis patients develop diabetes mellitus but the high frequency of association prompted us to explore whether mutations in *HFE* could increase the risk of developing diabetes mellitus in different populations.

The frequency of hemochromatosis gene variants was never studied in the Jordanian Arab population. Jordan contains a homogenous Arab population but received multiple migrations and population admixture. In this study we carried a population frequency analysis for *HFE* gene variants, C282Y, H63D, and S65C in 440 Jordanian Arabs. We also investigated the association of H63D variant with diabetes to determine if there is an increased frequency in diabetic patients from the same population.

2. Subjects and methods

2.1. Subjects

A total of 440 individuals have participated in this study. Subjects were recruited from individuals attending a major hospital that serves the north part of Jordan (King Abdullah University Hospital, Irbid). The largest group of subjects were blood donors with no abnormality as required by blood donors department (No diabetes, blood disorders, cardiovascular disorders, etc.). The other groups were patients attending the hospital, none of which had a hemochromatosis diagnosis. Classification of cases: 204 blood donors (age range: 18–49, average: 26.7 years), 89 type 2 diabetes (age range: 15–73, average: 54.7 years), 42 patients undergoing hemodialysis (age range: 11–81, average: 49.3 years), 29 thalassemia patients (age range: 2–30, average: 16.3 years), 29 patients attending the gynecology and fertility clinic (age range: 18–48, average: 33.4 years), 11 arthritis patients (age range: 23–59, average: 44.0 years) and 36 with miscellaneous indications including endocrine problems and autoimmunity. All 440 subject samples were used in the allele frequency analysis. Type 2 diabetes samples were used for the association analysis with blood donor samples as controls. Information regarding age, sex, and status of care needed were recorded. The mean age of population studied was 35.4 years, range 2–90 years. Positive control samples with homozygous mutants 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 participants or their legal guardians for children. This study was approved by the ethics committee (Institutional 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 [7]. Primers were designed using primer3 software [8]. Sequence 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 and S65C, same primer set was used: 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, 2X (dNTPs, MgCl2, 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 minutes at 95°C, the samples were processed through 35 temperature cycles of 30 seconds at 94°C, 30 seconds at 56°C, and 30 seconds at 72°C, and a final extension step of 72°C for 10 minutes. PCR products were run on 2% agarose gel and visualized by UV light after ethidium bormide staining to assess the correct sizing of the amplified product. Following PCR, products were digested by *RsaI* for C282Y, *MboI* for H63D and *HinfI* for S65C (Fermentas). The restriction fragments of PCR products were separated by electrophoresis on a 3% agarose gel containing 10 μg/ml ethidium bormide, and visualized by UV light and genotypes recorded. Positive and negative controls were included in each run.

Table 1
Mutant genotypes *HFE* (C282Y, H63D and S65C) and their frequency in the Jordanian population; WT denotes wild type

Genotype	Individuals with genotype	%
C282Y/WT	0	0
C282Y/C282Y	0	0
H63D/WT	93	21.1
H63D/H63D	3	0.68
S65C/WT	1	0.23
S65C/S65C	0	0
Compound heterozygous	0	0
WT/WT	343	78.0
Total	440	100

2.3. Statistical analysis

Data were entered in Excel (Microsoft Corporation) 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 variant locus. It was assessed in the observed genotype distribution with a Chi squared test. Allelic association p values were determined using a Chi squared test between cases and 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 [9]. 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.

3. Results

Subjects participating in the study were 440, 202 (45.9%) females and 238 (54.1%) males. Blood donors constituted the major group (204, 46.4%), the other subjects had different indications; diabetes mellitus (89, 20.2%), patients undergoing hemodialysis (42, 9.5%), thalassemia (29, 6.6%) patients attending the gynecology and fertility clinic (29, 6.6%), rheumatoid arthritis (11, 2.5%), and miscellaneous indications (36, 8.2%). Genotypes for the three *HFE* variants were analyzed in all subjects representing the north part of the Jordanian population. The frequencies of genotypes of C282Y, H63D, and S65C are shown in Table 1. C282Y was not found in all 440 individuals genotyped. S65C was found only in one case with an allele frequency of 0.11%. H63D was found to be a common allele in the Jordanian population with 93 heterozygotes (21.1%), three homozygote mutants (0.68%) and an allele frequency of 11.25%. No compound heterozygotes were found in our population. All genotypes were consistent with Hardy-Weinberg expectations.

Data were further divided into two groups of type 2 diabetic (89 subjects, 62% females) and blood donors (204 subjects, 28% females). Since only H63D has a considerable presence in the Jordanian population it was used to compare its frequency in type 2 diabetic population with blood donor population. The Blood donors represented the control population. H63D allele frequency of the blood donors (204 subjects) was 11.27%, which is comparable to H63D frequency (11.25%) in all 440 subjects. Genotype and allelic frequencies of the diabetic and blood donors control population are shown in table 3. No significant difference was found between H63D genotypes of the two groups ($p = 0.84$). Frequency of H63D heterozygous mutation in diabetic patients was 23.60% and in the control population 22.55%. No H63D homozygote mutant was present in either population. Allele frequency of the mutant H63D allele was 11.80% in the diabetic population and 11.27% in the control population with no significant difference ($p = 0.65$).

4. Discussion

This is the first study to analyze hemochromatosis gene variants, C282Y, H63D, and S65C in Jordanian Arab population. C282Y was not detected in all 440 samples tested. Frequency of H63D homozygous and heterozygous mutation was 0.68% and 22.1%, respectively. S65C was detected with 0.23% heterozygous and no homozygous. Furthermore, H63D variant was tested for association with type 2 diabetes patients. No significant association of H63D allele was found in our diabetes patients as compared to matched controls.

Jordan is a small country in the Middle East with a relatively homogenous population. It was part of the Roman Empire until the 7th century and received much influx of crusader armies in the 11th and 12th centuries. Thus, admixture of Europeans with the indigenous population might be expected to spread the C282Y variant that is thought to be generated north of Europe some 2000 years ago [10]. Unexpectedly, no C282Y allele was found in 440 individuals studied. This explains the rarity of hemochromatosis in Jordan. Total absence of C282Y in Jordanians could indicate that the genetic mixture between European crusaders and the Arab population was non-existent or negligible and that currently there is negligible genetic mixture with northern Europeans. Another explanation could be that the ma-

Table 2
Frequency of *HFE* variants in different populations as compared to the Jordanian population

Population	Allele frequencies %			Reference
	C282Y	H63D	S65C	
Bulgaria	0	23.0		[37]
Croatia	3.3	14.5	1.8	[28]
Denmark	5.6	12.8	1.8	[38]
Ireland, north	14	17.9		[4]
Italy, north	3.2	13.4	1.3	[29]
Italy, south	1.5	14.0	0.5	[30]
Finland	4.6	9.8	2.3	[31]
France	7.7	14.0	1.95	[18]
Portugal	2.8	23.0		[32]
Russia	3.7	13.3	1.7	[33]
Slovenia	4.0	14.5	0.5	[28]
Spain, north	3.0	20.0	1.0	[34]
Spain, central	2.0	16.0		[36]
Sweden	6.2	11.4	1.6	[35]
Sweden, Saami	2.0	7.9	3.0	[31]
Jordan	**0**	**11.25**	**0.11**	**Present study**
Saudi Arabia	0	8.5–17.7		[5,16]
Tunisia	0.5	17.5		[17]
USA, Caucasian	6.8	15.2	1.6	[11]
USA, Hispanic	2.7	12.4	0.6	[11]
USA, Asian	0.2	3.3	0	[11]
USA, African	1.1	5.1	1.7	[11]
Ecuador	0	3.5	4.0	[12]

jority of the crusaders were of southern European origin having a low frequency of the C282Y. Searching cases for hemochromatosis in the largest tertiary healthcare hospital in northern Jordan which serves 1.5 million inhabitants we found only one case of hemochromatosis. Testing the patient revealed a single H63D mutant allele.

Our results were compared to data reported from different populations. As shown in Table 2, the prevalence of C282Y variants is higher in North of Europe where the highest frequency is observed in areas between Nordic and Anglo-Saxon countries with rates fluctuating between 6% in Northern countries and 14% as reported for Ireland. In contrast, South of Europe, and in the Mediterranean countries the prevalence is very low. In countries such as Italy and France, highest frequency is located in the north (3.2%, 7.7% respectively) compared with the south (1.5%, 3.3%, the later value not shown in Table 2). In our study, C282Y frequency (0%) is less than those reported in Europe and USA but similar to those reported for countries of the Middle East and Africa (Table 2).

The S65C variant has not been investigated much and there is little information about its genetic frequency. There were no S65C homozygotes in our study and heterozygotes frequency was 0.23% of our population with an allele frequency of 0.11%. The lowest allele frequency was reported in Asian Americans [11] and the highest was reported in Ecuador [12]. S65C, when combined with C282Y, is associated with an increased risk of hemochromatosis diagnosis [13]. Recently, S65C variant was found to have a lowering effect on iron status markers [14].

H63D variant is more common than C282Y and the geographical distribution of H63D variant in Europe is the reverse of that observed for C282Y variant, following a decreasing line from south to north of Europe [15]. Most European populations being studied have an allele frequency of 10–20%. Frequency above 20% were observed in Spanish, Portuguese and Bulgarians with the highest frequency being reported for Basque population (30.4%) [5]. Our results show the H63D allele frequency to be 11.25% which seems higher than that found in Saudi Arabian population (8.5%) [5], on the other hand, a recent report in the Saudi population estimated allele frequency to be as high as 17.7% [16]. The 11.25% allele frequency in Jordan is closer to that reported from European populations like UK, Sweden, Norway and Italy [5]. In addition, Jordanian H63D allele frequency is much less than that reported in Spain where the highest European frequency is found and is slightly less than that reported for Turkey, Greece, and North Africa [5,17]. No compound heterozygotes were found. Thus our results agree with the fact that H63D variant is not restricted to the European population. Although this variant is associated with the mild form of hemochromatosis, it shows incomplete penetrance which decreases the clinical impact of this variant [18].

We attempted to test the association of the three *HFE* variants with diabetes, however, C282Y was completely absent from our study population and S65C was present in only one allele of the 880 alleles tested. H63D on the other hand was more frequent and allowed us to study its association with diabetes in the Jordanian Arab population. We did not find an association between H63D variant and diabetes in our cohort. This is consistent with a recent data from polish [19], Greek [20], British [21], American [22], Iranian [23], Australian [24], and Irish [25] populations. In contrast, one study in the Polish population found an increase in H63D allele carrier frequency in patients with diabetic nephropathy [26]. In a study in Spain, authors found increased frequency of H63D allele in patients of type 2 diabetes, however, they had marginal significance with a p value of 0.04, and a low number of control cases, 108 [27].

Diabetes is the most commonly observed endocrine disorder in people with hemochromatosis which is due

Table 3
Genotype and allele frequencies of H63D in tested individuals with Diabetes Mellitus and blood donors (controls)

	Diabetics		Blood donors		
H63D (c.187C > G)	N	%	N	%	*p* value
GG genotype	0	0	0	0	0.84
CG genotype	21	23.60	46	22.55	
CC genotype	68	76.40	158	77.45	
G allele	21	11.80	46	11.27	0.65
C allele	157	88.20	362	88.73	

to accumulation of iron which alters glucose and insulin homeostasis as seen in type 2 diabetes even if it is present in a lesser extent as observed in *HFE* heterozygotes. Since there was a repeated suggestion of an association between the inheritance of *HFE* variants and diabetes mellitus, and due to the high prevalence of diabetes in Jordan, it was rational to investigate if there is a relationship between H63D variant and having diabetes. However, there was no genetic difference between the control and the test group which suggests that H63D variant does not play a significant role in the development of diabetes mellitus in the Jordanian population and that other genetic and environmental factors may be considered. One limitation to mention is the lower age average in the controls as compared to cases. We re-analyzed the data considering half the controls, those with the higher age (102 control subjects, average age 33.0 years) and we still did not detect a significant association (data not shown).

In conclusion, we analyzed the frequency of the three hemochromatosis *HFE* variants; C282Y, H63D, and S65C in Jordanian Arab population. C282Y, the major disease causing mutation was completely absent from our 440 individuals tested. This is consistent with the fact that hemochromatosis has a very low prevalence in Jordan and that it is not merely under-diagnosed. S65C variant was found in a single allele. H63D variant was found to be common with 11.25% allele frequency, but it plays a minor role in hemochromatosis when compared to C282Y mutation. Furthermore, we analyzed the association of H63D variant with the risk of developing diabetes but we could not detect significant association in our test group suggesting that this variant does not play a major role in increasing the risk of developing diabetes in Jordanian Arab population.

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