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Correlation between *HFE* gene polymorphisms and increased risk of coronary artery disease among patients with type 2 diabetes in Iran

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Background/aim: Diabetes mellitus is a risk factor for cardiovascular diseases (CVDs), which are among the major causes of deaths in type 2 diabetes (T2D). The purpose of the present study was to determine the association of C282Y and H63D mutations in the *HFE* gene with increased risk of coronary artery disease (CAD) in T2D patients.

Materials and methods: Two hundred and ninety individuals were divided into two groups: a case group and a control group. Genomic DNA of peripheral venous blood cells was extracted and the *HFE* gene mutations were analyzed using the PCR–RFLP technique.

Results: Data analysis revealed a significant difference between the allele frequencies of H63D and C282Y mutations between the case group and the controls ($P < 0.05$). The relationships between the GA and GG genotypes in C282Y and H63D mutations in terms of fasting blood sugar (FBS), lipid profile (total cholesterol, triglycerides, high-density lipoproteins (HDL), low-density lipoproteins), body mass index (BMI), HbA1c, micro albuminuria, and creatine levels did not show a significant differences between the two groups ($P > 0.05$). Using a logistic regression model, BMI, FBS, HDL, and total cholesterol levels were significantly different with independent predictors of CVD ($P < 0.05$).

Conclusion: Our results revealed a significant correlation between C282Y and H63D mutations and the development of CAD in T2D patients

Key words: Type 2 diabetes, coronary artery disease, *HFE* gene, *H63D* and *C282Y* mutations

1. Introduction

It is well-established that diabetes mellitus is a risk factor for cardiovascular end points, which is the major cause of death in type 2 diabetes (T2D) (1,2). Several studies (3,4) have reported the association between higher iron storage and an increased risk of T2D mellitus. Many studies indicate that there are genes associated with increased risk of T2D mellitus, such as genes involved in iron metabolism {DEMİRBİLEK H , 2013 #19}(5,6). *HFE* gene variants were observed to be associated with iron overload (7,8). *HFE* gene variants cause hereditary hemochromatosis (HH), which often results in an increased risk of coronary artery disease (CAD) (9,10). It has been known for a long time that T2D mellitus accounts for 50% and 80% of patients with hemochromatosis (9), which causes an increased risk of CAD (10,11). Iron overload causes iron depots in some organs including the heart, which have been associated with CAD and reduced life expectancy (12).

Among the various genetic variants in the *HFE* gene region (13,14), 2 missense mutations, including a cysteine to tyrosine substitution at the amino acid position 282 (C282Y) and a histidine to aspartate substitution at the amino acid position 63 (H63D), have been the most investigated (7). The *HFE* gene product is a human leukocyte antigen-like molecule that is presented at the cell surface bound to β2-microglobulin, where it is thought to modify the affinity of transferrin for its receptor. These mutations are considered to be independent risk factors

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for diabetic nephropathy (2). Some studies also reported an increased risk of myocardial infarction (15) and CAD in C282Y and Cys282Tyr in carriers (12,16). Most published studies are cross-sectional, with a restricted number of potentially explanatory variables (13,15). Thus, considering the relationship between *HFE* mutations and the clinical features and complications of diabetes (such as lipid profile and body mass index (BMI) among others) is very important (2). In the present study, we aimed to assess whether or not mutations in the *HFE* gene might be associated with increased risk of CAD in type 2 diabetic patients. To our knowledge this is the first study conducted on Iranian population in this regard.

2. Materials and methods

This cross sectional case-control study is based on 290 individuals of Iranian ancestry. This population included patients with angina-like chest pain and noninvasive tests suggesting ischemia, indicating patients with depressed left ventricular ejection fraction of unknown origin and valvular heart disease. Patients with previously performed coronary bypass surgery or percutaneous transluminal coronary angioplasty were excluded from the analysis because of their treated coronary status. Participants who had a fasting blood glucose concentration >6.7 mmol/L, a blood glucose concentration of >10.0 mmol/L 2 h after a glucose load, and clinical diagnosis of diabetes requiring dietary, oral, or insulin treatment were considered as T2D. Patients with thyroid, liver, or renal diseases were excluded from the study. The patient group included 145 CAD patients with positive coronary angiograms (at least one vessel with >50% stenosis) with a history of T2D who attended the Shahid Chamran Hospital (Tehran University of Medical Sciences, Tehran, Iran).

The control group (with no evidence of CAD (no history of angina and normal resting ECG (17)) consisted of 145 individuals recruited from the general population in the same geographical region. All participants were matched for age and sex, with the number of women and men being similar in both groups. All participants gave written informed consent to the use of their blood for genetic analysis, and this study conformed to the principles of the Declaration of Helsinki. The local hospital ethics committee approved this study. At the time of blood sampling, patients were under their usual cardiovascular medications. Characteristics of the study population, including sex and age, in both groups are shown in Table 1.

After inclusion, clinical and laboratory data were collected for each patient from the clinical charts. The following variables were considered and analyzed in the present study: serum glucose, total cholesterol, high-density lipoproteins (HDL), low-density lipoproteins (LDL), triglycerides (TG), BMI, HbA1c, creatine (Cr), and microalbumin.

Table 1. Characteristics of the study population.

Characteristics	CAD subjects	Control subjects	P value
N (m/f)	72/73	71/74	0.831
Mean age (years)	53.9 ±9	51.3 ± 10	0.423
Mean weight (kg)	89.2 ± 3.8	86.3 ± 4.4	0.577
Systolic BP (mmHg)	139.5 ± 24.8	131.2 ± 19.8	0.001
Diastolic BP (mmHg)	80.2 ±11.8	83.6 ±9.4	0.002
Total cholesterol (mg/dL)	204.1 ±28.4	198.8 ± 34.1	0.001
BMI (kg/m^2)	29.4 ± 4.5	24.6 ± 2.6	0.756
FBS (mg/dL)	182.1 ± 20.3	189.6 ± 43.1	0.572
2 h plasma glucose (mmol/L)	9.1 ± 3.1	5.4 ± 7.4	0.012
Cr. (mg/dL)	1.02 ± 0.42	0.96 ± 0.2	0.031
TG (mg/dL)	112 ± 36.09	99 ± 40.4	0.112
HDL-C (mg/dL)	49.0 ± 9.7	45.0 ± 7.3	0.490
LDL-C (mg/dL)	103.0 ± 21.2	98.03 ± 12.5	0.471
HbA1c (%)	8.63 ± 1.2	8.23 ± 7.0	0.810
Microalbumin (mg/dL)	19.03 ± 6.6	11.12 ± 5.6	0.420

All data are expressed as a means (± SD), CAD: coronary artery disease; BMI: body mass index; FBS: fasting blood sugar, Cr: Creatine; TG: Triglycerides; HDL-C: high-density lipoprotein–cholesterol; LDL-C: low-density lipoprotein–cholesterol.

DNA was isolated from venous blood samples using Bioneer kit (Bioneer). To evaluate the prevalence of mutations in the *HFE* gene in the patient and control groups, PCR amplification and RFLP analysis were performed. PCR was performed for amplification of the C282Y and H63D sites using primers designed by Prime3plus software available on http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi. For H63D locus, the forward and reverse primers were 5'-ATGGTTAAGGCCTGTTGCTCTGTC and 5'-CCCTTGCTGTGGTTGTGATTTTC, respectively; for C282Y locus, the forward and reverse designed primers were 5'-TCCTCTTTCCTGTCAAGTGC and 5'-GATGACTCCAATGACTAGGG, respectively.

DNA was amplified under the following conditions: initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 61 °C (for H63D mutation) and 57 °C (for C282Y mutation) for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min. Then PCR amplification was followed by restriction enzyme cleavage: a 10 µL sample of the PCR product was digested in 20 µL reactions containing 10 U of *Rsa*I at 37 °C for the C282Y mutation and *Mbo*I for the H63D mutation for 3 h. The products were resolved on 8% polyacrylamide gel.

The statistical analyses were computed with SPSS (Version 16). Data were expressed as means and standard deviation (SD). All variables were tested by either one-way ANOVA or Student's t-test. Categorical variables were compared using the chi-square test. Variables with a significant difference between the cardiovascular disease (CVD) and non-CVD groups were evaluated by multiple logistic regressions with forward stepwise analysis to identify independent risk factors for CVD. Odds ratios with 95% confidence intervals (95% CIs) were calculated as an estimate of relative risk of CAD. The genotype at the C282Y and H63D sites of the HFE gene was divided as wild type and heterozygous or homozygous mutation. $P < 0.05$ was assumed to be significant.

3. Results

The prevalence of *HFE* mutations, including C282Y and H63D alterations in patients and controls, are shown in Table 2.

3.1. H63D mutations in HFE gene

Genotypes of H63D mutations were determined by incubating the PCR product with *Mbo*I restriction enzyme and resolving the obtained fragments on 8% polyacrylamide gel. The genotype and allele frequencies of H63D mutations are shown in Table 2. Two genotypes, CC and CG, were detected, but no homozygotes for the H63D mutation were found. The chi-square test showed a significant difference between the allele frequencies of H63D mutations among case groups and controls ($P < 0.05$). The frequency of CC and CG genotypes among the cases was 45% and 55%, respectively, and among the control subjects it was 66.7% and 33.3%, respectively. There was a significant difference in genotype distribution between the two groups ($P < 0.01$). We further analyzed the associations of the two genotypes with the physiologic variables shown in Table 3.

As shown in Table 3, for this mutation, two genotypes, CC and CG, were analyzed separately for each group. Studying the fasting blood sugar (FBS) values in the control group between the two genotypes showed that there was a significant difference between the two genotypes of H63D mutation ($P < 0.01$). The differences between the CC and CG genotypes in the patients group with respect to FBS were not significant ($P > 0.05$). Additionally, there was no significant association between the H63D mutation and plasma lipids levels (TC, TG, LDL-C, HDL-C). The difference in cholesterol levels between the two genotypes in the patient group was minimal ($P = 0.39$). Overall, no significant difference was observed in lipid profile parameters between the control and patient groups ($P > 0.05$). The relationship between the two genotypes in HbA1c, micro albuminuria, and Cr levels also showed no significant differences between the 2 groups ($P > 0.05$).

3.2. C282Y mutation in *HFE* gene

The *C282Y* mutation genotyping was done by incubating the PCR product with *Rsa*I restriction enzyme resolving the fragments on an 8% polyacrylamide gel.

The genotype and allele frequencies of C282Y mutation are shown in Table 4. The two heterozygote genotypes detected included GG and GA, but no homozygote for the C282Y mutation (AA) was found.

Table 2. Genotypic association of the H63D polymorphism with CAD.

Variable	Genotype (%)		OR (95% CI)	P-value
	CC	CG		
CAD subjects	45%	55%	1.17 5.09 (2.42)	0.01
Control subjects	66.7%	33.3%		

Table 3. Analysis of physiologic variables in the control and case groups.

Variable	CAD subjects			Control subjects		
	CG	CC	P-value	CG	CC	P-value
N	70	75	-	54	91	-
BMI (kg/m^2)	29 ± 4.4	29.5 ± 4.6	0.46	24.62 ± 4	24.58 ± 3.1	0.89
FBS (mg/dL)	178.1 ± 20.1	190.4 ± 2.3	0.17	186.2 ± 80.4	190.1 ± 20.9	0.01
Cr. (mg/dL)	0.9 ± 0.2	1 ± 0.4	0.18	1 ± 0.4	1.1 ± 0.3	0.38
TG (mg/dL)	107.9 ± 2.0	105 ± 2.0	0.95	77.3 ± 1.5	72.7 ± 1.4	0.7
Total cholesterol (mg/dL)	196.4 ± 31.8	187 ± 20.7	0.39	201.5 ± 10.6	198.10 ± 10.7	0.49
HDL-C (mg/dL)	51.8 ± 11.9	49.7 ± 11.9	0.28	41.5 ± 11.9	38.6 ± 10.3	0.13
LDL-C (mg/dL)	91.1 ± 33.4	87.5 ± 34.8	0.53	52.2 ± 14.1	50.9 ± 11.6	0.56
HbA1c (mg/dL)	8.3 ± 1.9	8.6 ± 2.0	0.39	7.9 ± 1.6	7.7 ± 1.5	0.67
Microalbumin (mg/dL)	21.1 ± 6.9	20.2 ± 6.8	0.47	6.7 ± 2.1	6.4 ± 2.0	0.41

All data are expressed as a means (± SD), CAD: coronary artery disease; BMI: body mass index; FBS: fasting blood sugar, Cr: Creatine; TG: Triglycerides; HDL-C: high-density lipoprotein–cholesterol; LDL-C: low-density lipoprotein–cholesterol.

Table 4. Genotypic associations of C282Y polymorphism with CAD.

Variable	Genotype (%)		OR (95% CI)	P-value
	GG	GA		
CAD subjects	35%	65%	1.03 4.34 (2.12)	0.03
Control subjects	53.3%	46.7%		

The frequency of GG and GA genotypes in the case group was 35% and 65%, respectively, while in the control group it was 46.7% and 53.3%, respectively. Statistical analysis by the chi-square test showed a significant difference between the allele frequencies of C282Y mutation among cases and controls ($P < 0.05$). There was a significant difference in genotype distribution among two groups ($P < 0.01$), suggesting there was a significant relationship between this genotype distribution and CAD status. In estimating risk of CAD in the presence of the GA genotype, as compared with the GG genotype, the heterozygote GA genotype was associated with a significantly higher risk of CAD ($P < 0.01$) (adjusted odds ratio (OR) = 2. 12, 95% confidence interval (CI) = 1.03–4. 34).

Investigation of the associations between the two genotypes and physiologic variables are shown in Table 5.

Similar to the results obtained from statistical analysis of H63D mutation, differences between the GA and GG genotypes in the C282Y mutation in patient and control groups were not significant. In addition, the relationship between the two genotypes in terms of FBS, lipid profile (TC, TG, LDL-C, HDL-C), BMI, HbA1c, micro albuminuria, and Cr levels showed no significant differences in the control and patient groups. It could be concluded that there is no relationship between these physiologic variables and the C282Y mutation status in both groups.

The results of stepwise multiple logistic regression showed that the independent risk factors for CVD were BMI, FBS, HDL, and total cholesterol level, and the patients had all these risk factors (Table 6).

4. Discussion

To our knowledge, this is the first study on the evaluation of whether mutations in the *HFE* gene might be associated with increased risk of CAD in T2D patients in Iranian population. Our result revealed a significant difference between the allele frequencies of H63D and C282Y mutations. This genotype distribution suggests a significant association between *HFE* mutations and CAD status. The C282Y heterozygote and homozygote frequencies were higher in this study than in other reported study. In addition, our results indicate that *HFE* mutations are genetic markers for CAD risk in the Iranian

Table 5. Analysis of physiologic variables in the control and case groups.

Variable	CAD subjects			Control subjects		
	GA	GG	P-value	GA	GG	P-value
N	86	59	-	70	75	-
BMI (kg/m^2)	29.3 ± 4.1	29.2 ± 5.1	0.97	24.6 ± 3.3	24.4 ± 3.7	0.71
FBS (mg/dL)	183.1 ± 20.2	187.7 ± 20.3	0.36	189.4 ± 90.3	187.9 ± 8.9	0.33
Cr. (mg/dL)	0.9 ± 0.3	1 ± 0.3	0.16	1.1 ± 0.4	1 ± 0.3	0.42
TG (mg/dL)	107.1 ± 2.0	105.3 ± 2.0	0.76	76.2 ± 1.4	72.3 ± 1.5	0.29
Total cholesterol (mg/dL)	197.4 ± 20.8	194.5 ± 20.8	0.42	175.6 ± 20.7	177.4 ± 20.6	0.19
HDL-C (mg/dL)	50.8 ± 12.1	50.6 ± 11.7	0.94	38.9 ± 11.2	40.4 ± 10.8	0.41
LDL-C (mg/dL)	88.4 ± 34.4	90.4 ± 33.8	0.72	50.5 ± 13.2	52.3 ± 12.3	0.39
HbA1c (%)	8.48 ± 2.0	8.46 ± 2.0	0.95	7.8 ± 1.6	7.8 ± 1.5	0.84
Microalbumin (mg/dL)	20.5 ± 6.3	20.8 ± 7.6	0.80	6.4 ± 2.0	6.6 ± 2.0	0.44

All data are expressed as a means (± SD), CAD: coronary artery disease; BMI: body mass index; FBS: fasting blood sugar, Cr: Creatine; TG: Triglycerides; HDL-C: high-density lipoprotein–cholesterol; LDL-C: low-density lipoprotein–cholesterol.

population. The incidence of diabetes is globally increasing and considered as an epidemic. It shows the importance of investigations in this study due to macrovascular complications that individuals with this condition may experience and consequently CVDs. On the other hand, CVDs are the most prevalent causes of mortality and morbidity among people with diabetes (18).

Diabetic patients aggregate other comorbidities such as obesity, dyslipidemia, and hypertension, which also contribute to an increased risk for CVDs. It has been stated that diabetes acts as an independent risk factor for CVD in both men and women (19). Several forms of CVDs are listed as the cause of death in 65% of patients with diabetes. It could be expected that the frequency of *HFE* gene mutations could be elevated among T2D patients and as a result, among CAD cases (19,20). The HFE protein is a type I transmembrane protein bound to β2-microglobulin, and the mutated HFE protein at position 63 or position 282 can result in the lack of association with β2-microglobulin, thereby disrupting the association of *HFE* with transferrin receptor, which leads to increased iron absorption (7).

Table 6. Stepwise multiple logistic regression analysis of factors associated with CVD in patients with T2D (n = 145).

Risk factors [a]	OR (95% CI)	P-value [b]
BMI (kg/m^2)	1.12 (1.21–1.04)	0.036
FBS (mg/dL)	0.90 (0.98–0.83)	0.023
Total cholesterol	2.78 (1.04–4.52)	0.036
HDL-C (mg/dL)	1.43 (1.02–1.78)	0.002

[a]Included factors: body mass index (BMI), fasting blood sugar (FBS), total cholesterol, high-density lipoproteins (HDL), low-density lipoproteins (LDL), triglycerides (TG), HbA1c, creatine (Cr), and microalbumin. OR: ratio; CI: odds confidence interval.
[b]Based on Wald c2 statistic.

The identification of *HFE* variants (C282Y and H63D) provides the unique opportunity to test whether genetic polymorphisms that are associated with tissue iron accumulation may also be considered as CAD risk factors (18). Some types of *HFE* genotypes (C282Y/H63D or heterozygous C282Y carriers) are associated with increased risk of different disorders like hereditary hemochromatosis and diabetes (10). Our paper shows that the differences between the CC and CG genotypes in the patient group in regards to FBS were not significant ($P > 0.05$) because PGC-1α participates in lipid metabolism and the plasma lipid levels were stratified according to these two SNP variants.

The association of *HFE* gene mutations and the risk of T2D has been extensively studied in different populations (9,21,22). However, the results of these investigations were inconsistent (7). For example, in a study by Surber et al. (23), the C282Y mutation was not associated with an increased risk of CAD or severity of coronary lesions. This study did not find a linkage between the C282Y mutation and previous myocardial infarction or depressed left ventricular ejection fraction (23). In an angiographically controlled population, ferritin and

transferrin concentrations were not associated with CAD (24). In a population (265 patients) with proven premature CAD a lower frequency of the C282Y mutation, compared with healthy controls, was found (25). Therefore, this result leads us to longitudinal studies with large samples of these mutations to study its association with CAD in diabetic patients.

To obtain an estimate of the prevalence of the C282Y and H63D mutations in the Iranian population, we conducted a nested cross-sectional study of 145 incident cases of CAD with a history of T2D and 145 matching control subjects. We then examined the prevalence of the 2 mutations in case and control subjects. A significant difference between the allele frequencies of H63D and C282Y mutations among cases and controls was observed. This genotype distribution suggests a significant association between *HFE* mutations and CAD status. Study on Italian (26), United States, and United Kingdom (9) populations indicated the possible contribution of *HFE* mutations to iron status disorders, and consequently to CAD risk. In a subset of patients, indexes of physiologic variables including FBS, total cholesterol, HDL, LDL, TG, BMI, HbA1c, and grand microalbumin was not associated with cardiovascular end points. It could be concluded that there is no relationship between these physiologic variables and HFE gene status or CAD as a diabetic complication. The reason for the lack of this association could be related to the small sample size in our study. The results showed that the prevalence of CVD and insufficient control of CVD risk factors among our patients were high. These findings are in agreement with other studies in different regions (27,28). More aggressive interventions are crucial for patients with diabetes mellitus, including better patient education and more aggressive control of glycaemia, hypertension, hyperlipidemia, and metabolic syndrome.

In conclusion, our findings provide evidence of an association between C282Y and H63D mutations and the development of CAD in diabetic patients, thus indicating that *HFE* mutations are genetic markers of CAD risk in Iranian population. Similar conclusions were reached by Carolina Pardo Silva et al. (12), in a study in which they reported significant association between *HFE* gene mutations and increased risk of CAD in women but not in men.

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