

Original Research

Association of *HFE* gene mutations with nonalcoholic fatty liver disease in the Iranian population

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Abstract: To determine whether the *HFE* gene variants H63D and C282Y are associated with NAFLD in persons with type 2 diabetes, we conducted a case-control study including 145 case of NAFLD patients with a history of type 2 diabetes and 145 matching control. The genomic DNA was extracted from the peripheral venous blood and the genotyping of *HFE* gene mutations was analyzed using the PCR-RFLP technique. Statistical analysis was performed using SPSS 12.0 software by χ^2 test, t test and ANOVA ($P < 0.05$). Data showed no increased frequency of *HFE* mutations in persons with type 2 diabetes and no association between H63D mutation and NAFLD in the study population. Also, we analyzed index of physiological variables including FBS, lipid profile (TC, TG, LDL-C, and HDL-C), BMI, HbA1c, and micro albuminuria and Cr levels). Data showed there are no relationship between these indexes and *HFE* gene mutations and either NAFLD as a complication of diabetes. But our results showed a relationship between C282Y mutation and NAFLD in persons with type 2 diabetes. C282Y mutation might be a genetic marker of NAFLD in Iranian population.

Key words: Type 2 diabetes, Nonalcoholic fatty liver disease, *HFE* gene, H63D, C282Y mutations.

Introduction

The role of iron overload in the pathogenesis of type 2 diabetes mellitus is currently discussed. However the exact link between iron and the development of diabetes remains unknown. Progressive tissue accumulation of iron leads gradually to the damage of the liver, heart, endocrine organ, skin and muscles (1). Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease (2), The prevalence of NAFLD has increased with the epidemics of type 2 diabetes, which is risk factor for NAFLD (3). Iron overload influences lipid and glucose metabolism and it has been reported in NAFLD and could contribute to pathogenesis and progression of NAFLD (4-8).

There are several candidate genes linked with iron metabolism and other pathways have been associated with risk of type 2 diabetes mellitus (11-9). Among which *HFE* gene variants were observed to be associated with iron overload (12). The *HFE* gene was first discovered as the causative gene for hemochromatosis in 1996. The *HFE* gene product is an HLA-like molecule that is presented at the cell surface bound to b2-microglobulin, where it is proposed to modify the affinity of transferrin for its receptor (13). Among several genetic variants in the *HFE* gene region (14,15), 2 missense mutations including a cysteine to tyrosine substitution at amino acid position 282 (C282Y) and a histidine to aspartate substitution at amino acid position 63 (H63D) have been the mostly investigated and these are the most iron loading genotypes (12,16-19).

It has been known for a long time that between 50% and 80% of patients with hemochromatosis have type 2 diabetes. Therefore, it could be expected that the

frequency of *HFE* gene mutations could be increased among type 2 diabetes patients. Several studies have been performed to show the association between these two conditions but the overall interpretation of these studies is that the presence of *HFE* mutations is not a major cause of type 2 diabetes (20-25).

On the other hand, Since NAFLD and *HFE* mutations are common, many authors studied the association between these two conditions and found conflicting results. Such as, a wide scope meta-analysis study showed a positive association for some genotypes with NAFLD (26). But a systemic review and meta-analysis study does not support an association between the *HFE* genetic variants and the presence of NAFLD (27).

In the present study we aimed to assess whether C282Y and H63D mutations in the *HFE* gene might be associated with increased risk of NAFLD in Iranian patients with type 2 diabetes.

Materials and Methods

This case-control study is based on 290 individuals of Iranian ancestry from May 2014 to May 2016. The patients group included 145 patients with biopsy-proven NAFLD with a history of type 2 diabetes attended the

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Shahid Chamran Hospital in Tehran, Iran. All Patients were recruited by means of liver biopsy. We analyzed only the patients with a negative history of alcohol consumption and other known causes of chronic liver disease (e.g. viral hepatitis, autoimmune hepatitis, use of hepatotoxic medications such as glucocorticoids, antibiotics, tamoxifen or other anti-neoplastic drugs (28-30). Patients in whom liver ultrasound examination was not available were excluded from the study. The control group was based on 145 individuals representing a general population sample from the same geographical region. All participants were matched for age and sex and women and men were equal in both groups respectively. All participants gave written informed consent to the use of their blood for genetic analysis, and this study conforms to the principles of the Declaration of Helsinki. The local hospital ethics committee approved this study. Characteristics of study population including gender and age of patient and control groups are shown in Table 1.

Clinical and laboratory data were collected for each patient from the clinical charts including: Serum glucose, total cholesterol, high-density lipoproteins (HDL), Low-density lipoproteins (LDL), Triglycerides (TG), body mass index (BMI), HbA1c, Creatine (Cr) and Micro albumin.

DNA was isolated from blood (Bioneer's DNA Extraction -USA). The *HFE* genotype was determined by PCR amplification and RFLP analysis with *RsaI* for the C282Y mutation and with *MboI* for the H63D mutation (31-32). Design of primers was performed using Prime3plus software available on <http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>. The Sequence of primers and length of PCR products are represented in table 2.

PCR Conditions

Thermal cycling was carried out as follows: 95°C for 5 minutes, then 30 cycles of 95°C for 30 seconds, annealing at 61.2°C (for H63D mutation) and 57.2°C (for C282Y mutation) for 30 s, extension at 72°C for 30 s and a final extension at 72°C for 5 min. The PCR amplification was tested by running 10 µL of the product on a 2% agarose gel.

Restriction Enzyme Analysis

Digestion was performed by incubation 8.5 µL of

Table 1. Characteristics of the study population.

Characteristics	Patients	Controls
Total	145	145
Woman	72	72
Man	73	73
Mean age	53.9 ± 9	51.3 ± 10

Table 2. Information of sequence of primers, length of PCR products and product size of restriction enzymes.

Name of gene	Name of mutations	Sequence of primers	PCR product length	Enzyme	Size of restriction product
<i>HFE</i>	(H63D) rs:799945	F: ATGGTTAAGGCCTGTTGCTCTGTC	294	<i>MboI</i>	138
		R: CCCTTGCTGTGGTTGTGATTTTC			99
					57
<i>HFE</i>	(C282Y) rs:1800562	F: TCCTCTTTCCTGTCAAGTGC	489	<i>RsaI</i>	245
		R: GATGACTCCAATGACTAGGG			244

PCR products with 0.5 µl of enzyme at 10U in a final reaction volume of 10 µL at 37°C for 3 h. Restriction fragments were separated by 2% agarose gel in TBE buffer for 1h at 100 v. Gel was stained with ethidium bromide (0.5 µg/ml) and photographed by ultra violet photography.

Statistical analyses were performed using SPSS 12.0 software (SPSS, Chicago, IL, USA). Frequencies of the genotypes and alleles were compared using the χ^2 test. Two-tailed independent sample t-tests were performed to detect differences between groups. Odds ratios (ORs) and their 95% confidence intervals (CIs) were evaluated. The relations between mutations in the *HFE* gene and clinical and biochemical variables were evaluated by ANOVA. The criterion for significance was set at $P < 0.05$ for all tests. Data are presented as mean ± standard deviation.

Results

We need to mention that all data are in Hardy-Weinberg equilibrium.

The H63D mutation

Genotype of H63D mutation was determined by incubation of PCR product with *MboI* restriction enzyme. The gel has been shown in Figure 1. Genotypes and allele frequencies of H63D mutation are shown in Table 3. Three genotypes were detected. The Chi-square test showed no significant difference between the allele frequencies of H63D mutation among cases and controls. There was not a significant difference in genotype distribution between the two groups. Also, we analyzed relation of the genotypes with physiological variables including: FBS, lipid profile (TC, TG, LDL-

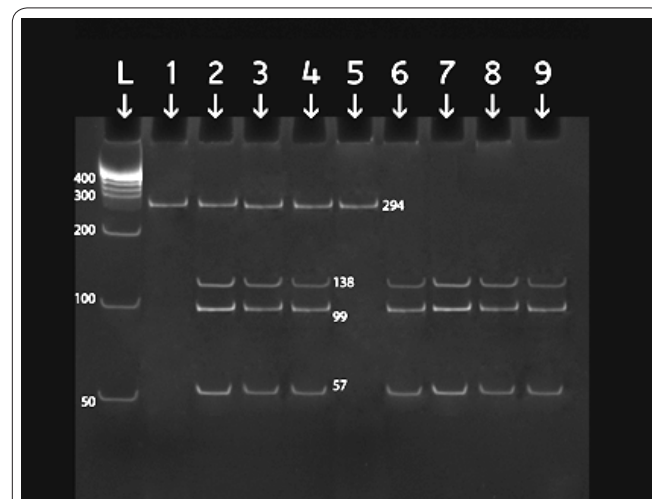


Figure 1. Genotypes of H63D mutation. L: Ladder 50bp; 1 and 5: GG genotype (294bp); 2-4: CG genotype (294,138,99,57bp); 6-9: CC genotype (138,99,57bp).

Table 3. Allele and genotype frequencies of the H63D mutation among NAFLD patients and control subjects.

Allele/Genotype	Patients	Controls	P value	OR (CI)
Alleles	n=290	n=290		
C	227	233	0.539	0.881(0.59-1.318)
G	63	57		
Genotypes	N=145	N=145		
CC	85	90	0.548	0.866(0.541-1.386)
CG	57	53	0.628	1.124(0.699-1.807)
GG	3	2	1	1.511(0.249-9.177)

Table 4. Analysis of physiological variable in case and control groups. (According to H63D mutation).

Variable	Patients				Controls			
	P-value	CG	CC	GG	P-value	CG	CC	
N	-	57	85	3	-	53	90	
BMI	0.46	29±4.4	29.5±4.6	29±2	0.89	24.62±4	24.58±3.1	24.60±1
FBS	0.17	78.1±2.1	90.4±2.3	80±2.1	0.01	86.2±8.4	90.1±2.9	88.5±2
Cr	0.18	0.9±0.2	1±0.4	0.92±0.3	0.38	1±0.4	1.1±0.3	1±0.1
TG	0.95	107.9±2	105±2	106±2	0.7	77.3±1.5	72.7±1.4	75±1
Chol.	0.39	46.4±1.8	46±1.7	46.2±1.6	0.49	44.5±1.6	48.1±1.7	46.1±1
HDL-C	0.28	51.8±11.9	49.7±11.9	50±11.9	0.13	41.5±11.9	38.6±10.3	40±5
LDL-C	0.53	91.1±33.4	87.5±34.8	88.4±34	0.56	52.2±14.1	50.9±11.6	52±10
HbA1c	0.39	8.3±1.9	8.6±2	8.4±1.9	0.67	7.9±1.6	7.7±1.5	7.8±1
Micro albumin	0.47	21.1±6.9	20.2±6.8	20.8±6.8	0.41	6.7±2.1	6.4±2	6.5±2

NAFLD: Nonalcoholic fatty liver disease; BMI: body mass index (BMI below 27 was considered as the lean and obese. BMI greater than or equal to 27 was considered as fat); LDL-C: low-density lipoprotein-cholesterol; HDL-C: high-density lipoprotein-cholesterol; TG: Triglycerides; Cr: Creatine; Chol:Cholesterol.

C, and HDL-C), BMI, HbA1c, micro albuminuria and Cr levels (Table 4). The Results showed that there were no significance differences between genotypes in two-groups in physiological variables.

The C282Y mutation

Genotype of C282Y mutation was determined by incubation of PCR product with *RsaI* restriction enzyme. The gel has been shown in Figure 2. Genotypes and allele frequencies of C282Y mutation are shown in Table 5. Three genotypes were detected. Chi-square test statistic showed a significant difference between allele frequency of C282Y mutation in case and control

groups. Therefore, there is a significant relationship between genotype distribution of C282Y mutation and NAFLD, And GG genotype is associated with a significantly increased risk of NAFLD. ($p=0.003$) (Adjusted odds ratio (OR) = 2.84, 95% confidence interval (CI) = (1.288-3.372). Also, we analyzed relation of the genotypes with physiological variables including: FBS, lipid profile (TC, TG, LDL-C, and HDL-C), BMI, HbA1c, micro albuminuria and Cr levels (Table 6). The results showed there were no significance differences between genotypes in two groups in physiological variables.

Discussion

To determine whether the *HFE* gene variants H63D and C282Y are associated with NAFLD in persons with type 2 diabetes, we conducted case-control study including 145 case of NAFLD patients with a history of type 2 diabetes and 145 matching control. Our finding showed no increased frequency of the *HFE* mutations in persons with type 2 diabetes. Our results showed no association between H63D mutation and NAFLD in persons with type 2 diabetes. Also results showed a relationship between C282Y mutation and NAFLD in the same population. Because of *HFE* gene participates in lipid and glucose metabolism (4-8), we analyzed index of physiological variables including FBS, lipid profile (TC, TG, LDL-C, and HDL-C), BMI, HbA1c, microalbuminuria and Cr levels). But data showed there are no relationship between these indexes and *HFE* gene mutations and either NAFLD as a complication of diabetes. It is to be mentioned, to our knowledge; this is the first

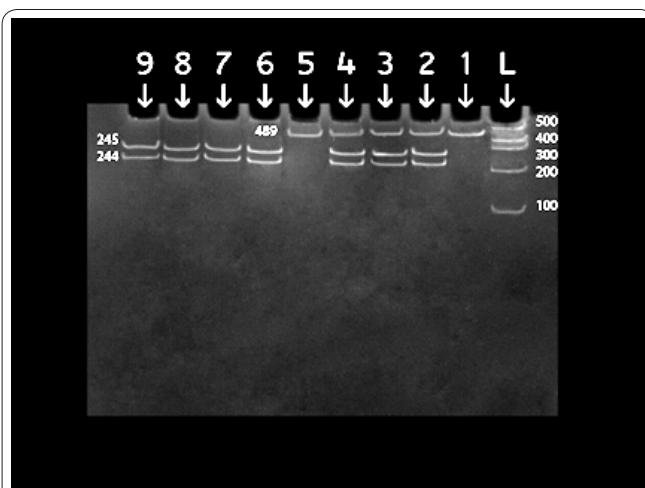


Figure 2. Genotypes of C282Y mutation. L: Ladder 100bp; 1 and 5: AA genotype (489bp); 2-4: GA genotype(489,245 and 244 bp); 6-9:GG genotype (245,244bp).

Table 5. Allele and genotype frequencies of the C282Y mutation among NAFLD patients and control subjects.

Allele/Genotype	Patients	Controls	P value	OR (CI)
Alleles	n=290	n=290		
G	244	216	0.004	1.817(1.205-2.741)
A	46	74		
Genotypes	N=145	N=145		
GG	101	76	0.003	2.84(1.288-3.372)
GA	42	64	0.000003	0.322(0.198-0.524)
AA	2	5	0.251	0.392(0.075-2.052)

Table 6. Analysis of physiological variable in case and control groups. (According to C282Y mutation).

Variable	Patients				Controls			
	P-value	GA	GG	AA	P-value	GA	GG	
N	-	42	101	2	-	64	76	5
BMI	0.97	29.3±4.1	29.2±5.1	29.1±3	0.71	24.6±3.3	24.4±3.7	24.3±3.4
FBS	0.36	83.1±2.2	87.7±2.3	89.3±2.1	0.33	89.4±9.3	87.9±8.9	88.3±9
Cr	0.16	0.9±0.3	1±0.3	0.92±0.3	0.42	1.1±0.4	1±0.3	1.1±0.3
TG	0.76	107.1±2	105.3±2	106.1±2	0.29	76.2±1.4	72.3±1.5	75.3±1.5
Chol.	0.42	47.4±1.8	44.5±1.8	45.1±1.9	0.19	45.6±1.7	47.4±1.6	46.5±1.5
HDL-C	0.94	50.8±12.1	50.6±11.7	50.9±10	0.41	38.9±11.2	40.4±10.8	39.1±10
LDL-C	0.72	88.4±34.4	90.4±33.8	89.3±32	0.39	50.5±13.2	52.3±12.3	51.1±12
HbA1c	0.95	8.48±2	8.46±2	8.3±2	0.84	7.8±1.6	7.8±1.5	7.8±1.2
Micro albumin	0.8	20.5±6.3	20.8±7.6	20.6±5.5	0.44	6.4±2	6.6±2	6.46±2

study to date to determine the association between these two conditions in the Iranian population with diabetes.

Several studies have been performed to show the association between *HFE* mutations and type 2 diabetic. While some studies reported an increased frequency of *HFE* mutations in persons with type 2 diabetes (22-20), other studies failed to detect such association (25-23). Although, we mentioned Pooled analysis of these studies tends not to support that the presence of *HFE* mutations is a major cause of type 2 diabetes. Our finding were consistent across with studies that failed to detect such association (23-25). A quite different issue is whether the presence of *HFE* alleles could effect on the clinical expression of type 2 diabetes. There is a growing body of work showing that iron and glucose metabolism are interdependent and that an increased iron store may contribute to IR. Persons with type 2 diabetes with *HFE* mutations have been described to have increased iron parameters (33). Serum iron may contribute to IR via increased adipocyte lipolysis and impairment of glucose transport (34). There is evidence from in vitro studies that iron can reduce binding of insulin to its receptor and reduce insulin receptor gene expression (35).

On the other hand, family studies and inter-ethnic variations in susceptibility suggest that genetic factors may be important in determining disease risk or the clinical course of NAFLD. A number of studies have examined the association between variants H63D and C282Y and the risk of NAFLD and found conflicting results. The first report about the connection between *HFE* gene mutations and NAFLD showed a positive correlation between levels of serum ferritin, iron, and transferrin saturation and the presence of the mutated allele, as well as between C282Y mutation and more severe fibrosis in a North American population (36). The

results of George et al. supported these findings (37). A wide scope meta-analysis published suggested a positive association for some genotypes with NAFLD such as c282y 2007 (26). That is consistent with our observation.

In contrast Bugianesi et al. (38) and other studies found no association between *HFE* gene mutations and NAFLD (39-41). Also, another meta-analysis and systematic review does not support an association between the *HFE* genetic variants and the presence of NAFLD and they believed the *HFE* may have none or, at maximum, a marginal role in the development of NAFLD (27).

A cohort polish study showed neither the C282Y nor the H63D *HFE* gene mutations were associated with NAFLD pathogenesis, and no differences between frequencies of *HFE* gene mutations in subgroups of NAFLD patients with less and more severe liver fibrosis. In polish study, 16.1% of study population had type 2 diabetes mellitus (DMt2). Also they found that in subgroup of NASH patients with severe fibrosis, there were statistically significant correlations between the presence of at least one mutated allele of H63D and total cholesterol as well as LDL-cholesterol levels (42). In our study compared with polish study, all of patients have type 2 diabetes mellitus. And stages of disease was not specified. Also we found no correlation between cholesterol levels and *HFE* gene mutation.

Generally, the conflicting evidence regarding *HFE* gene mutation and NAFLD could be the result of the different ethnic structure, Inadequate sample size, referral and ascertainment biases, lack of mediators' adjustment, or publication bias could explain such in consistencies(27).

Some studies suggested an association between the

presence of the C282Y mutation and other *HFE* genotypes predisposing to iron overload with more severe liver damage (43-44). A defect of our study was no determining of stage and severity of NAFLD in patients. And also we didn't determine the relation of *HFE* gene Mutations specially C282Y with these two factors. Another defect of our study was that we didn't determine blood parameters related to iron overload and also didn't determine relation of these factors with *HFE* gene Mutations. It's better if it was be done.

Regardless of being or not being association between NAFLD and HFE mutations, since one of the genes that considered with hereditary hemochromatosis is *HFE* gene (45), also the *HFE* mutations H63D and C282Y are an important cause in iron overload(46,47). There is a suggestion that the effect of increased iron absorption on fat deposition development in liver may extend to liver diseases including NAFLD (48).

Finally, Future studies should focus on the role of *HFE* gene in the progression of NAFLD and its association with iron biomarkers (peripheral and hepatic), and also these results should be validated in more meta-analysis study and larger racially heterogenic populations of NAFLD patients.

In conclusion our findings showed an association between C282Y mutation and NAFLD in Iranian population with diabetes, thus C282Y mutation might be a genetic marker of NAFLD in Iranian population. Also, we found no association between H63D mutation and NAFLD in person with Type 2 diabetes in Iran.

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