Hemochromatosis gene (*HFE*) mutations in patients with type 2 diabetes and their control group in an Iranian population

Faranak Sharifi, MD, *Abdulreza Esmaeilzadeh,* PhD, *Mohammadreza Zali,* MD.

ABSTRACT

الأهداف: من أجل تقييم تكرار شكلين اثنين من طفرة الجين الصباغي الدموي HEF، وطفرة *C282Y*و*H63D* لدى السكان السليمين بالمقارنة مع المرضى المصابين بداء السكري من النوع الثاني T2DM.

الطريقة: أجريت هذه الدراسة في مركز زانجان لمرض السكر، بإقليم زانجان – إيران، في عام 2005م. شملت الدراسة 202 فرداً: 101 من المرضى المصابين بداء السكري من النوع الثاني T2DM، و101 فرداً كمجموعة تحكم مطابقة لنوع الجنس والعمر. تم فحصهم من أجل طفرة الجين الصباغي الدموي HFE. تم تكبير مضاد نيوكلوتيد 845 *C282Y* و187 *H63D* بواسطة تفاعل سلسلة الخمائر الناقلة PCR. تم تحليل منتج تفاعل سلسلة الخمائر الناقلة PCR بواسطة تقييد أنزيم الهضم. إستخدمنا اختبار تشي–سكوير واختبار فيشر للمقارنة، وتم حساب معدل (Odds) البيانات الحاصلة.

النتائج: تمت دراسة 202 فرداً. بلغ التكرار الواسع لمضاد *C282Y*-98/2% لدى المرضى المصابين بداء السكري من النوع الثاني T2DM، وبلغ 99/1% في مجموعة التحكم (p=0.6). بلغ التكرار الواسع لمضاد *H63D*-68.3/31.7% في المرضى المصابين بالسكري (p=0.08) و73.4/26.3% في مجموعة التحكم (p=0.08)، على التوالي. لم يكن توزيع نوع الجين مختلفاً بشكل ملحوظ.

خاتمة: بناءً على بياناتنا لطفرة الجين الصباغي الدموي HEF، لم يتبين وجوده بشكل مفرط لدى المرضى المصابين بداء السكري من النوع الثانيT2DM، وليس هنالك دليل يشير بفرط هذه المضادات في النوع الثاني من داء السكري T2DM بناء على البحث المبني على السكان.

Objective: To assess the frequency of 2 different forms of hemochromatosis (HFE) gene mutations (*C282Y* and *H63D* mutations) in a normal population in comparison with type 2 diabetic patients.

Methods: This case control study was undertaken in Zanjan Diabetic Care Center, Zanjan, western Tehran, in 2005. Two hundred and two individuals were included in this study: 101 type 2 diabetes mellitus (T2DM) patients, and 101 age, and gender-matched controls. The patients were examined for mutations in the HFE gene. Nucleotide 845 (*C282Y*) and 187 (*H63D*) alleles were amplified by polymerase chain reaction (PCR) with lymphocyte deoxy-ribonucleic acid. The PCR products were analyzed by restriction enzyme digestion. Chi-square, student's t test, and Fisher's exact tests were used for comparison, and odds' ratio was calculated.

Results: Two hundred and two individuals were studied. The frequency of wild/*C282Y* alleles was 98/2% in T2DM patients, and 99/1% in controls (p=0.6). The frequency of wild/*H63D* alleles was 68.3/31.7% in diabetics (p=0.08), and 73.4/26.3% in control subjects (p=0.08). The distribution of genotypes was not statistically different.

Conclusion: Based on our data, HFE mutations were not found in excess in patients with T2DM, and there was no evidence that a population-based search for an excess of these alleles in type 2 diabetes was indicated.

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From the Department of Endocrinology (Sharifi), Zanjan Metabolic Diseases Research Center, and the Department of Molecular Immunology (Esmaeilzadeh), Zanjan University of Medical Sciences, Zanjan, Iran, and the Department of Gastroenterology (Zali), Research Center for Gastroenterology and Liver Disease, Tehran, Iran.

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Address correspondence and reprint request to: Dr. Faranak Sharifi, Associate Professor, Department of Endocrinology, Vali-e-asr Hospital, Zanjan Metabolic Diseases Research Center, Zanjan, Iran. Tel. +98 (241) 7270815. Fax. +98 (241) 4262486. E-mail: faranaksharifi@hotmail.com

Hereditary hemochromatosis (HH) is one of the most common genetic disease of Caucasians,[1-3]and is an autosomal recessive condition, in which excess iron is absorbed by the intestine, and accumulates as excess tissue iron over many years.[4] Although effective treatments for HH can cause normal life expectancy, iron overload is dangerous for the body.[5] Although HH can be detected via biochemical blood analyses and liver biopsies,[6] these methods may be unreliable in some cases.[7,8] The discovery of the hemochromatosis HFE (*HLA-H*) gene has provided a sensitive test for the chromosome 6-linked component of hemochromatosis.[9] Two disease-linked alleles were described. The allele that is of major importance in symptomatic HH, is a G-to-A transition of nucleotide 845 of the c-deoxy-ribonucleic acid (DNA), which causes a cysteine 282 to tyrosine amino acid change in the predicted protein. A second, less-penetrant, allele is a C-to-G change of nucleotide 187 that causes a histidine 63 to aspartic acid change. This mutation is in complete linkage disequilibrium with the 845A mutation (in other words, no chromosome 6 with the 845A mutation carries the 187G mutation). However, the 187G mutation is found in excess only in HH patients when present with the 845A mutation as a compound heterozygote (heterozygosity for both 845A and 187G). Most patients with HH (87-100%) are homozygous for the *C282Y* allele, or are compound heterozygotes for these mutations.[2,3,9] Hereditary hemochromatosis was recognized as a cause of diabetes mellitus,[10] and there are some reports of the prevalence of HH with insensitive markers in subjects with T2DM.[11-13] The potential role of serum ferritin in the pathogenesis of type 2 diabetes has become interesting in recent years.[14,15] Most studies have revealed elevated ferritin concentration in the diabetic group of patients, and pre diabetes situations in comparison with normal subjects.[16-18] This evidence suggest that excessive ferritin concentration can be a marker of iron overload, and sub clinical hemochromatosis in diabetic patients. Is the high serum ferritin in type 2 diabetic patients the consequence of mutations in HFE gene? It was not revealed. Iron overload was revealed to decrease insulin sensitivity,[15,19] and can cause earlier complications in diabetes mellitus.[20] As we have no sufficient data regarding the frequency of mutant HH gene in Iran, this study was designed to search for an association of HFE mutations with T2DM in the population of Zanjan, north-western Iran.

Methods. One hundred and one patients with type 2 diabetes, including 70 female and 31 males were recruited serially from the Zanjan Diabetes Centre, Zanjan, Iran, in 2005. Patients included were more than 20 years of age at the time of diagnosis, and were not taking oral hypoglycemics for at least 6 months. Insulin treatment was not a reason for exclusion, as long as the patients were without insulin, 6 months after diagnosis. Patients with type 2 diabetes resulting from chronic pancreatitis, or steroid use were excluded. In families with more than one diabetic patient, one of them was randomly included in the study, and others were excluded. One hundred and one control subjects were age- and gender-matched healthy people, who came for check up without known diabetes. Peripheral venous blood sampling was undertaken for lymphocyte DNA and iron studies. Each patient was classified as one of 6 HFE genotypes, as follows: 1) *845G/845G* homozygote (normal), 2) *845A/845A* mutant homozygote (potentially affected), 3) *845G/845A* heterozygote 4 with *187C/187C* (normal), 5) *187G/187G* homozygote (potentially affected); (6) *187C/187G* heterozygote. Approval from the ethics committee of Zanjan University of Medical Sciences was obtained. All participants were informed regarding the aims of the study, and informed consent was obtained from all of them.

DNA amplification. Genomic DNA was extracted by salting out method,[26] and amplified by polymerase chain reaction (PCR). A PCR reaction was performed in 25 ml volume with 12 pmol of *HFE* exon 2 primers (5'-ACATGGTTAAGGCCTGTTGC-3' and 5'-GCCACATCTGGCTTGAAATT-3'), and another PCR with 12 pmol of *HFE* exon 4 primers (5'-CCTTCCTCCAACCTATAGAA-3' and 5'-CAGCCCATCCCCTAACAAAG-3').[27] For both, we used 50-100 ng of genomic DNA, 1 unit Taq DNA polymerase (Super Taq, HT Bioscience, UK), 0.2 mM of each *dATP, dGTP, dCTP, dTTP* (HT Bioscience, UK), 2 mM magnesium chloride, and 1 x PCR buffer (50 mM potassium chloride, 20 mM Tris-hydrochloric acid, pH 8.4). After an initial temperature of 94°C for one minute, 35 amplification cycles were performed on a personal thermocycler (Eppendorf, Gmbh, Hamburg, Germany). Each cycle consisted of denaturation at 94°C for 20 seconds, annealing at 55°C (*C282Y*), and 58°C (*H63D*) for 20 seconds, and extension at 72°C for 40 seconds. A final extension step (72°C for 10 minutes) terminated the process. To verify proper amplification conditions, 5 µl of each PCR product from the amplification of the HFE exons were then analyzed electrophoretically on an ethidium bromide-stained 1% agarose gel at 100 volts (V) for 40 minutes. The amplification of exon 2

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(for *H63D* mutation)were detected by the presence of bands at 208 base pair (bp), and exon 4 (for *C282Y* mutation) were detected by the presence of bands at 486 bp. To determine the polymorphism of exon 2, 5 µl of amplified DNA was digested with one µl of BclI (10u/µl) restriction endonucleases (Fermentas, Germany) treated with one µl of buffer G^+ (Fermentas, Germany) at 55°C for 3 hours, and to determine the polymorphism of exon 4, 5 µl of amplified DNA was digested with 0.5 µl of RsaI (10u/µl) restriction endonucleases (Fermentas, Germany) treated with one µl of buffer Y^+ (Fermentas, Germany), at 37°C for 3 hours. Ten percent polyacrylamide gel electrophoresis at 150 V for 120 minutes was used to separate restriction fragments. For HFE gene codon 63, bands at 138, and 70 bp after endonuclease digestion revealed a wild type allele, characterized by the presence of BclI restriction site. The presence of a single band at 208 bp indicated homozygous mutant alleles, characterized by the absence of BclI restriction site. The heterozygous alleles were characterized by the presence of bands at 208, 138, and 70 bp. Polymorphism in codon 282 was investigated after RsaI digestion by the presence or absence of bands at 193 and 164 bp. For this codon, mutant allele contains 2 RsaI sites (GTAC) to split PCR product into fragments of 293, 164, and 29 bp, whereas wild type allele was characterized by the absence of one site, and was revealed by presence of bands at 293 and 193 bp. Homozygosity for tyrosine at codon 282 was determined by the presence of bands at 293 and 164 bp (the 29 bp band is often not visualized), whereas 3 bands at 293, 193, and 164 bp indicated heterozygosity.

Data analysis. Statistical analysis was conducted using SPSS software (version 11.5). Proportions were compared using the Chi-square test. Group means were compared by using student's t test. Differences in allele frequencies between groups were assessed with the Fisher's exact test. Odds ratio was calculated for the prevalence of HFE mutations in diabetic patients and for both genders. Significance at $p<0.05$ was taken for 2-sided tests.

Results. Two hundred and two people, including 141 females and 61 males were recruited in this study. Genotyping failed in 6 of the blood samples, and finally we reported the genotype of 196 subjects. **Table 1** presents general characteristics and biochemical data of the diabetic and the control group. The mean serum iron in subjects with mutant *H63D* alleles was 90±29 µg/l, and was not different from participants without this mutation (90±37 µg/l, $p=0.5$). The serum iron concentration in subjects with *C282Y* mutant alleles was not different from those without this mutation either (105±48 µg/l versus 88±36 µg/l, $p=0.3$). None of the 101 patients, and none of the 101 control subjects were *845A/845A* mutant homozygote (C282Y) ($p=1.0$). Three of the 202 participants (1.5%, 95% (confidence interval (CI): 0-3%) including 2 of the diabetic patients (2%, 95% CI: 0-4%), and one of the control group (1.1%) were *845G/845A* mutant heterozygote ($p=0.5$). Five of the 202 people participating in this study (2.6%, 95% CI: 0.4-4%) were homozygous for the *H63D* mutation (*187G/187G* homozygote). All the subjects

Table 1 - General characteristics and biochemical markers of diabetic and control group.

Parameters	Diabetic group (n=101)	Control group (n=101)	*p*-value
Age, years	55.4±11.4	55.2±11.3	0.9
Male/female	31/70	30/71	0.5
Duration of diabetes, years	6.5±5.9	-	-
FPG, mg/dl	160±40	80±14	0.01
HbA1c, %	7.8±2.1	-	-
Serum iron, mg/dl	91.3±38.9	84.7±34	0.2
Hb, g/dl	12.9±0.5	13.5±1.2	0.1

FPG - fasting plasma glucose, HbA1c - glycosated hemoglobin, Hb - hemoglobin

Table 2 - Frequency of different types of HFE mutations in males and females (n: males=60, females=136).

Frequencies of mutation	Gender		Total
	Female	Male	
Frequency of H63 mutation			
Non, n (%)	100 (73.5)	39 (65)	**139**
Heterozygous n (%)	33 (24.3)	19 (31.7)	**52**
Homozygous n (%)	3 (2.2)	2 (3.4)	**5**
Frequency of C282Y mutation			
Non, n (%)	134 (98.5)	59 (98.3)	**193**
Heterozygous, n (%)	2 (1.5)	1 (1.7)	**3**
Homozygous, n (%)	-	-	-

Table 3 - Clinical and biochemical data in homozygotes or heterozygotes for mutant alleles of *HFE* gene in comparison with normal genotype subjects (N=196).

Genotype	Age, years	Frequency of diabetes (%)	Serum iron concentration (mg/dl)
845G/845A	48.3±6.6	66.7	105±48
187G/187G	52.6±13.8	100	90.6±29
187C/187G	56.6±11.3	51.9	84±35.8
No mutation	55±11.4	49.6	88±36

p-value is non-significant, continous variables expressed as mean±SD

with this type of mutation were diabetic (5%, 95% CI: 0.8-9.2%), and none of the control group were mutant in this type (p=0.08).

Fifty-two of the 202 participants (25.7%, 95% CI: 20.4-32.6%) were heterozygote for mutation *H63D* (*187C/187G* heterozygote) including 27 diabetic (26.7%, 95% CI: 16.6-36.8%), and 25 normal (24.7%) subjects (p=0.8). Odd's ratio for mutation H63D in diabetes mellitus was estimated to be 1.3 (95% CI: 0.6-2.4, p=0.4) and for mutation *C282Y* was 1.9 (95% CI: 0.16-21, p=0.6). **Table 2** presents the frequency of the mutations in diabetic and control people in terms of their gender. **Table 3** illustrates the clinical and biochemical data in homozygotes or heterozygotes for mutant alleles of HFE gene in comparison with normal genotype subjects.

Discussion. The prevalence of the HFE mutation in the Iranian population was not previously reported. We did not detect an excess of HFE mutations in patients with T2DM as compared with matched control subjects without known diabetes in this study. Most of the previous studies using biochemical or tissue markers in patients with diabetes have failed to detect an excess of HH,[23,24] although there are exceptions.[25,26] Some of the methods used in these studies have poor diagnostic accuracy,[11,27] and glycemic control may have an influence on some of these measurements.[27] The present study, using a more specific genetic marker for mutations in the HFE gene, has failed to demonstrate any significant excess in the mutations in patients with T2DM. The prevalence of *845A* homozygosity of 0/101 in our population with T2DM was identical to the age- and gender-matched control subjects. Despite the small sample size in our study, our results for the frequency of *845A* homozygosity is very close to the frequency of 1/212 observed in a population of diabetic patients in the United Kingdom.[28]

No excess of HFE 845A homozygosity in T2DM has been reported in different populations recently.[29-35] When available data from these studies[14,29-38] are combined with the present data, the overall prevalence of HFE *845A* homozygosity becomes 0.46% in T2DM, as compared with 0.365% in those without diabetes (p=0.76). Although the majority of these studies (and the present study), have not shown an excess of mutant alleles in the type 2 diabetes populations, there are some exceptions. Kwan et al[33] studied 105 patients with T2DM, and 103 patients with type 1 diabetes as a control group. Although there was no excess of *845 (C282Y)* homozygotes in the type 2 population, 22 heterozygotes were present. A borderline significant increase in the frequency of this allele in the type 2 patients (odds ratio 2.1; 95% CI: 1-4.5; p=0.048) was detected. Some researchers pointed out, that these data in a small sample should be interpreted cautiously. In another report from Fernandez-Real et al,[34] in 170 Spanish patients when compared with 108 control subjects without diabetes, a borderline significant increase in the *H63D* allele frequency was detected (odds ratio 1.17; 95% CI: 1.01-1.36; p=0.04). The frequency of the *H63D* mutation as reported in this study was lower than the frequency of 26.3%, reported for a different Spanish population.[2] This finding may in part be explained by the small control group. We have found that neither homozygosity nor heterozygosity for the *H63D* mutation was elevated in an Iranian diabetic population, as compared with results in control subjects. One previous observation in England revealed, that the penetrance of the HFE gene with respect to disease manifestations may be low in the local population as most of the HFE *845A* homozygotes are not diagnosed with the manifestations of hemochromatosis.[3] In another study, patients carrying at least one *C282Y* allele had a younger age of onset of diabetes as well as a longer duration of the disease (p=0.007).[39] We could not detect an earlier age of onset in type 2 diabetic patients carrying HFE mutations in our study. We did not search for diabetes complications in this study, however, an increased prevalence of retinopathy (p=0.014), and of nephropathy (p=0.04) were detected in individuals carrying at least one C282Y allele in some other studies.[39-41]

In our study, the 2 *845A* heterozygous patients identified in the group with diabetes did not have elevated iron levels, suggesting the probable low penetrance of the HFE gene with respect to biochemical manifestations in this population. In one previous study,[19] T2DM patients, females in particular, with mono-allelic *C282Y* mutation, had slightly increased iron parameters. In that study, no difference in insulin sensitivity or B cell function was observed in the presence of mono-allelic HFE mutations. The treatment of hemochromatosis may retard the progression of T2DM[42] it is still necessary to consider it for individual T2DM patients who may warrant screening, such as those with a family history of hemochromatosis, those with an associated cardiomyopathy or gonad failure, and perhaps those with abnormal liver function tests.

In conclusion, the present data from Iranian population, and other similar reports from other countries do not support population-based genetic screening of patients with T2DM, to detect the alleles *C282Y* and *H63D*.

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