Screening of the HFE Gene Mutations in Turkish Patients with Cryptogenic Cirrhosis and Hemochromatosis

Kriptojenik Siroz ve Hemokromatozisli Türk Hastalarda HFE Gen Mutasyon Taraması

Saffet ÖZTÜRK,[a]
Hilmi DİKİCİ, MD,[b]
Dinç DİNÇER, MD,[b]
Güven LÜLECİ,[a]
İbrahim KESER[a]

Departments of
[a]Medical Genetics,
[b]Gastroenterology,
Akdeniz University Faculty of Medicine, Antalya

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Yazışma Adresi/*Correspondence:*
İbrahim KESER
Akdeniz University Faculty of Medicine,
Department of Medical Genetics,
Antalya,
TÜRKİYE/TURKEY
keser@akdeniz.edu.tr

ABSTRACT Objective: The aim of this study was to determine the prevalence of the HFE gene mutations (C282Y, S65C and H63D) in patients with cryptogenic cirrhosis, hemochromatosis and healthy controls. **Material and Methods:** The exon 2 and exon 4 of the HFE gene were amplified by polymerase chain reaction (PCR) in the DNA samples of 18 cryptogenic cirrhotic and 11 hemochromatosis patients, and 141 healthy control individuals.Then the restriction fragment length polymorphism (RFLP) method was used to detect the mutations. **Results:** The frequencies of C282Y, S65C and H63D mutations were found as 0.0, 0.0, 0.12 respectively in healthy Turkish population and 0.0, 0.0, and 0.11 respectively in cryptogenic cirrhotic patients. We also screened 11 hemochromatosis patients for these mutations, and the frequencies of the mutations were found as 0.0 for C282Y, 0.0 for S65C, and 0.27 for H63D mutation. There was no difference between the control group and cryptogenic cirrhosis group. However, we found differences in the frequency of the H63D mutation between the control group and hemochromatosis group. **Conclusion:** The frequencies of the C282Y and S65C mutations were found as 0.0 in Turkish population and in the patients with cryptogenic cirrhosis similar to other Asian populations. However, the frequency of the H63D mutation was higher than previously reported in Asian populations. These results suggest that the H63D mutation may be responsible for the hereditary hemochromatosis in Turkish population.

Key Words: Liver cirrhosis; hemochromatosis; DNA mutational analysis; polymerase chain reaction; restriction mapping

ÖZET Amaç: Bu çalışmanın amacı, kriptojenik sirozlu, hemokromatozisli hastalarda ve sağlıklı kontrol bireylerde HFE gen mutasyonları (C282Y, S65C ve H63D) sıklığının belirlenmesiydi. **Gereç ve Yöntemler:** On sekiz kriptojenik sirozlu ve 11 hemokromatozisli hasta ile 141 sağlıklı kontrol bireyin DNA örneklerinde HFE geninin ekzon 2 ve ekzon 4 polimeraz zincir reaksiyonu (PCR) ile çoğaltıldı.Sonra, mutasyonları saptamak için restriksiyon fragmantı uzunluk polimorfizmi (RFLP) yöntemi kullanıldı. **Bulgular:** C282Y, S65C ve H63D mutasyonlarının sıklığı sağlıklı Türk popülasyonda sırasıyla 0.0, 0.0, 0.12 ve kriptojenik sirozlu hastalarda sırasıyla 0.0, 0.0, 0,11 olarak bulundu. Ayrıca, 11 hemokromatozisli hastayı bu mutasyonlar açısından taradık ve mutasyon sıklıkları C282Y için 0.0; S65C için 0,0; ve H63D mutasyonu için 0,27 olarak bulundu.Kontrol grubu ve kriptojenik siroz grubu arasında fark yoktu. Ancak, kontrol grubu ve hemokromatozisli grup arasında H63D mutasyon sıklığı açısından farklılıklar bulundu. **Sonuç:** C282Y ve S65C mutasyon sıklıkları diğer Asya popülasyonlarında olduğu gibi Türk popülasyonu ve kriptojenik sirozlu hastalarda 0.0 olarak bulundu.Ancak, H63D mutasyon sıklığı daha önce Asya popülasyonlarında rapor edilenlerden daha yüksekti.Bu sonuçlar H63D mutasyonunun Türk popülasyonunda kalıtsal hemokromatozisten sorumlu olabileceğini göstermektedir.

Anahtar Kelimeler: Karaciğer sirozu; hemokromatozis; DNA mutasyon analizi; polimeraz zincir reaksiyonu; restriksiyon haritalaması

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Cryptogenic cirrhosis has an unidentifiable etiology despite many diagnostic advances in hepatology. Cryptogenic cirrhosis constitutes 5-30% of the cirrhotic patients. The relation between HFE gene mutations and cryptogenic cirrhosis with iron metabolism disorder has not been well defined.[1]

The HFE gene, identified by positional cloning in 1996 by Feder et al., is responsible for hereditary hemochormatosis.[2] Three missense mutations (C282Y, S65C and H63D) have been described on the HFE gene in patients suffering from hemochromatosis on the basis of phenotypic data.[3] The major mutation, C282Y, is a G→A transition at nucleotide 845 of the open reading frame that changes the 282 cysteine to tyrosine. It accounts for 80% to 90% of hereditary hemochromatosis chromosomes in the world. The second mutation, S65C, a substitution in exon 2, altering the 65 amino acid serine to cysteine, has been associated with the development of a mild form of iron overload. The third mutation, H63D, is a C→G transition in codon 63, resulting in the substitution of histidine for aspartic acid. Because of its high frequency in the general population, its role in the disease remains uncertain. This variant could indeed be a minor mutation causing iron overload with a low penetrance.[4,5]

The aim of this study was to screen the HFE gene mutations (C282Y, S65C, and H63D) as a genetic factor in the patients with cryptogenic cirrhosis as compared to the patients with hemochromatosis and the healthy controls.

MATERIAL AND METHODS

PATIENTS

Eighteen cryptogenic cirrhotic patients (10 females, eight males; mean age: 49.06 years, range: 19-72) and 141 randomly selected healthy volunteers (92 females, 49 males; mean age: 31.01 years, range: 19-61) who were living in Antalya were included in the study. Additionally, we screened 11 patients with hemochromatosis. Patients with crytogenic cirrhosis selected after excluding known reasons of cirrhosis including hepatitis virus infections (HBV and HCV), excessive alcohol consumption, alpha-1-antitrypsin deficiency, autoantibodies such as anti-smooth muscle antibody, anti-nuclear antibody and anti-mitochondrial antibody, Wilson's disease, and hemochromatosis. Hemochromatosis patients were included into the study according to the absence of secondary causes of iron overload (anemia, thalassemia major, or dietary iron overload), transferrin saturation greater than 50% and ferritin levels greater than 400 ng/ml. Patient and control groups completed a detailed health questionnaire including demographic data, and signed an informed consent that was approved by the Ethical Committee of Akdeniz University Medical School. Genomic DNA was isolated from peripheral blood cells according to the protocol described previously.[6]

The HFE gene mutations, C282Y, S65C and H63D, were determined by the polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP) technique using specific primers and restriction enzymes. The same primers were used for S65C and H63D mutations. The primers of the PCR amplifications for the C282Y, mutations were designed as C282Y-forward 5'-TGG CAA GGG TAA ACA GAT CC -3' and C282Y-reverse 5'-CTC AGG CAC TCC TCT CAA CC -3'. The S65C and H63D primers were H63D/S65C-forward 5'-ACA TGG TTA AGG CCT GTT GC -3', and H63D/S65C-reverse 5'-GCC ACA TCT GGC TTG AAA TT -3'.[2,7]

We used a total volume of 50 ml per reaction which included 10x PCR buffer (160 mM $(NH4)_2SO_4$, 670 mM Tris HCl pH 8.8, 0.1% Tween-20), 20 mM $MgSO_4$, 20 mM each primers, 100 mM dNTP mix, 250 ng template DNA, 5 U/ml Taq DNA polymerase (Bioron and BioGen). The PCR was carried out in a thermal cycler (Techne Genius). Amplification conditions consisted of 10 minutes initial denaturation at 95 ^{0}C followed by 40 cycles of denaturation at 95 ^{0}C for 1 minute, annealing at 55 ^{0}C for 1 minute and extension at 72 ^{0}C for 1 minute, the final extension at 72 ^{0}C for 10 minutes. The fragment sizes for C282Y and H63D/S65C were 388 bp and 200 bp, respectively. The PCR products were digested with the restriction enzymes *Rsa I*, *Hinf I* and *Mbo I (Nde II)* (Roche) at 37 ^{0}C to identify the C282Y, S65C and H63D mutations, respectively. The restriction fragments of

the PCR products were separated by 3% agarose gel electrophoresis stained with ethidium bromide and photographed under UV light.

Serum iron concentration and total iron binding capacity (TIBC) were measured by photometric technique with autoanalyser. Transferrin saturation (TS) was calculated as the ratio of serum iron concentration to TIBC. Serum ferritin measurements were performed by ECL technique in the Central Laboratory of Akdeniz University Medical School.

RESULTS

A total of 18 cryptogenic cirrhotic patients (10 females, eight males) over the age of 19 years (range: 19-72 years; mean: 49.06 years,) and 141 healthy control subjects (92 females, 49 males) over the age of 19 years (range: 19-61 years; mean: 30.01 years) were evaluated for C282Y, H63D and S65C of the HFE gene mutations. The allelic frequencies of the mutations determined in the patient group and in the control subjects are given in Tables 1 and 2. All of the patients and the control group were found to be homozygous wild type for C282Y and S65C mutations. One heterozygous (5.56%) and one homozygous (5.56%) for the H63D mutation was found in the patient group. Among 141 control subjects, two (1.42%) were homozygous and 30 (21.28%) were heterozygous for the H63D mutation. Additionally, two of the 11 patients with hemochromatosis were heterozygous; other two were homozygous for the H63D mutation. Neither the C282Y nor S65C were found in the hemochromatosis patients. The allelic frequency of the H63D showed significant differences between hemochromatosis (27%) and cryptogenic chirrhosis (8.33%) patients and the controls (12.06%). The H63D genotypes and biochemical iron parameters of the patient and control groups are summarized in Table 3.

TABLE 1: Genotypes of the HFE gene mutations in the cryptogenic cirrhotic patients and in the healthy Turkish individuals.

HFE Mutations	H63D			S65C			C282Y		
Genotype	N/N	N/M	M/M	N/N	N/M	M/M	N/N	N/M	M/M
Patients	16	1	1	18	0	0	18	0	0
Control Subjects	109	30	2	141	0	0	141	0	0

N: Normal allele, M: Mutant allele.

TABLE 2: The genotype frequencies of the H63D mutation and allelic frequencies of the HFE gene mutations in the patients with cryptogenic cirrhosis and the control groups.

HFE Genotype	Cryptogenic Cirrhosis n (%)	Control Group n (%)
H63D/H63D	1 (5.56)	2 (1.42)
H63D/N	1 (5.56)	30 (21.28)
N/N	16 (88.88)	109 (77.30)
Alelle frequency of the C282Y mutation	0	0
Alelle frequency of the H63D mutation	8.33	12.06
Alelle frequency of the S65C mutation	0	0

N: Normal allele

The patients who were homozygous for the H63D mutation had higher ferritin, serum iron concentration and transferin saturation values when compared to normal and heterozygous individuals for the same mutation with the exception of total iron binding capacity. Interestingly, a patient who had cryptogenic cirrhosis and heterozygous for H63D had lower ferritin, serum iron level and transferritin saturation than homozygous normal control individuls. Examination of the patient's records showed that the patient developed esophageal varices in grade IV and antral gastritis according to the results of esophagoscopy and gastroscopy, respectively.

DISCUSSION

In this study, we report the results of screening for HFE gene mutations (C282Y, S65C and H63D) in 18 patients with cryptogenic cirrhosis and in 141 healthy controls from Antalya, Turkey. The frequencies of these HFE gene mutations in the cryptogenic cirrhosis were compared with to hemochromatosis and control groups. Although many screening studies related to hereditary hemochromatosis have been performed in different populations, there is a few population-based studies in Turkish population in the different perspectives.[8-11] The results of these studies show that C282Y and S65C mutations are very rare or absent in Turkish population.[8-10] However, it has been reported that the frequency of the C282Y mutation is high in people of Angloceltic origin.[2,12,13] This mu-

TABLE 3: The mean biochemical parameters in the cryptogenic cirrhotic patients and the control subjects in terms of normal, heterozygous and homozygous genotypes for the H63D mutation.

Iron Parameters	Cryptogenic Cirrhotic Patients			Control Subjects		
	N/N (n= 16)	H63D/N (n= 1)	H63D/H63D (n= 1)	N/N (n= 109)	H63D/N (n= 30)	H63D/H63D (n= 2)
Ferritin (ng/ml)	94.08 ± 7.0	4.76	159.60	27.24 ± 4.49	15.73 ± 4.06	130.50 ± 15.14
SIC (µg/dl)	84.64 ± 10.46	31.00	170.00	84.20 ± 11.36	73.50 ± 8.91	160.00 ± 26.28
TIBC (µg/dl)	264.64 ± 13.72	312.00	176.00	342.40 ± 16.58	384.50 ± 17.11	269.50 ± 13.44
TS (%)	31.98 ± 8.45	9.94	96.59	24.59 ± 6.09	19.12 ± 6.06	55.95 ± 3.56

N: Normal allele, SIC: Serum iron concentration, TIBC: Total iron binding capacity, TS: Transferrin saturation.

tation is also absent in populations of Asia.[14,15] The results of the present study showed the absence of the C282Y and S65C mutations and high prevalence of the H63D mutation in both cryptogenic cirrhosis and hemochromatosis and control individuals.However, Lal et al. secreened the C282Y mutation in 22 and 26 patients diagnosised as cryptogenic cirrhosis and hepatitis C cirrhosis, respectively.[16] They found one heterozygous case for the C282Y mutation among the crptogenic cirrhosis patients. On the other hand, three of the 26 hepatitis C cirrhotic cases were detected as heterozygous mutant for the C282Y mutation.[16]

The importance of being homozygous and heterozygous for H63D is not well understood. In this study, the frequency of the H63D mutation was found to be higher than other Asian populations with 12.06%.[14,15] Therefore, the H63D mutation may play a role in expression and progression of the cryptic cirrhosis and hereditary hemochromatosis, if it is associated with intrinsic and extrinsic factors in Turkish population.

Iron metabolism is regulated by genetic, physiological, and environmental factors. In our study, the patients with cryptogenic cirrhosis who were homozygous for the H63D mutation have had higher ferritin, serum iron concentration and transferin saturation than normal and heterozygous individuals for H63D mutation. The reason of low level ferritin, serum iron concentration and transferrine saturation of the patient that have heterozygous H63D mutation may be related to the loss of blood from esophageal varices. In literature, there are differetent studies performed to determine association between cirrhosis and HFE gene mutations.[17-20] Willis et al. have secreened the HFE gene mutations (C282Y and H63D) in 190 cirrhotic patients using polymerase chain reaction.[18] Of the 190 patients with cirrhosis, five (2.6%) patients and six of the cases (3.1%) were found to be homozygous mutants for the C282Y mutation and C282Y/H63D compound heterozygous, respectively. These mutations were significantly more frequent than the control group.[18] In our opinion, if more cases would have been investigated, the H63D mutation would have been related to iron parameters in the diseases such as cryptogenic cirrhosis and hemochromatosis.

In conclusion, the results of our study suggest that the C282Y and S65C mutations of the HFE gene are rare or absent in Turkish population. The frequency of the H63D mutation is higher in normal population of Turkey than other Asian populations. For example, according to the study performed by Lin et al., allelic frequency of H63D mutation was 2.3% in normal Chinese Han population.[21] The investigators secreened the three HFE gene mutations in 395 normal individuals.[21] The allele frequencies of the H63D mutation among healthy control subjects were 7.8% in Korea, 3.2% in Tailand, 0.99 in Japan.[22-24] There is no clear evidence that H63D mutation leads to iron overload related to several diseases in some of the Asian populations.[25-27] In other investigations from the USA, Denmark, Canada, and Germany, H63D homozygotes were reported to have a high risk of having increased iron levels.[28-31] Cogswell et al. found that transferrin saturation of the healthy people homozygous mutant for H63D aged 50 years or older had increased risk of iron overload than the younger ones (12-49 years).[32]

As a result, this mutation (H63D) may be responsible for the cryptogenic cirrhosis and hemochromatosis. The subjects carrying the H63D mutation should be periodically followed up in hepatology departments.

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