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Haplotype Analysis of Hemochromatosis Gene Polymorphisms in Chronic Hepatitis C Virus Infection: A Case Control Study

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Abstract

Background: Chronic hepatitis C virus (HCV) infection is frequently associated with elevated serum iron markers. Polymorphisms in the hemochromatosis (*HFE*) genes are responsible for iron accumulation in most cases of hemochromatosis, and may play a role in HCV infection.

Objectives: We aimed to assess the prevalence of *HFE* gene polymorphisms in a group of Iranian HCV-infected patients, and to explore the association of these polymorphisms with HCV infection.

Patients and Methods: *HFE* gene polymorphisms were examined in a total of 69 HCV patients and 69 healthy controls using polymerase chain reaction and restriction fragment length polymorphism techniques. Haplotype and diplotype analyses were performed using PHASE software.

Results: In a recessive analysis model of the His63Asp (H63D) locus (HH vs. HD + DD), the HH genotype was more common in patients compared to controls (adjusted P = 0.012; OR = 6.42 [95% CI: 1.51 - 27.33]). Also, in a recessive analysis model of the Cys282Tyr (C282Y) locus (CC vs. CY + YY), the CC genotype was more frequent in patients compared to controls (adjusted P = 0.03; OR = 5.06 [95% CI: 1.13 - 22.06]). In addition, there was a significant association between the HC haplotype and the HCDC diplotype and HCV infection.

Conclusions: Polymorphism in the hemochromatosis gene may confer some degree of risk for HCV infection, and individuals carrying the H and C alleles may be susceptible to this disease; however, a larger sample of HCV patients and healthy individuals may be necessary to further illustrate the role of these polymorphisms in HCV.

Keywords: Hepatitis C Virus, Hemochromatosis (*HFE*) Gene Polymorphisms, Prognosis

1. Background

Hepatitis C virus (HCV) infection is one of the most common chronic blood infections throughout the world, with approximately 170 million individuals affected worldwide (1). The role of iron in regulating viral hepatitis was first described by Blumberg et al. (2). They found that a high level of serum iron or ferritin caused spontaneous improvement of HCV infections, and the disease was limited after a severe infection. Increased levels of serum ferritin and serum saturated iron percentages in patients with chronic hepatitis C is usually observed (3-5). With the discovery that polymorphisms in the hemochromatosis (*HFE*) gene account for most cases of hereditary hemochromatosis, there has been much interest in their role in the development and progression of other liver diseases. Contradictory results have been reported with regard to the relationship between viral hepatitis infections and polymorphisms of the *HFE* gene. Past studies have proved that the prevalence of a polymorphism of Cys282Tyr (C282Y), the most significant polymorphism in the *HFE* gene related to hereditary hemochromatosis, is frequently reported in chronic hepatitis C patients (6-8). Although *HFE* gene polymorphism carriers are frequently seen among hepatitis C patients, the effect of *HFE* gene polymorphisms on HCV infection remains unknown. The *HFE* gene product is a major histocompatibility complex type I protein, and many immunologic variations have been found due to polymorphisms in this gene. Variations may induce different immunologic host responses in patients with *HFE* gene poly-

morphisms and concurrent viral hepatitis (9-11).

Hemochromatosis is a result of at least two polymorphisms in the *HFE* gene (previously known as HLA). The *HFE* gene is located on the short arm of chromosome 6 (12). The C282Y polymorphism, in which a G nucleotide is changed to an A at nucleotide position 845 of the *HFE* gene, results in a substitution of cysteine amino acid with a tyrosine residue at codon 282 and the alpha-3 domain of the *HFE* gene product. The His63Asp (H63D) polymorphism, in which a C is changed to a G at nucleotide position 187 of the *HFE* gene, results in a replacement of amino acid 63 at the alpha-3 domain, which removes a histidine residue and substitutes it with an aspartic acid (13, 14). Studies have demonstrated that the C282Y polymorphism prevents the *HFE* gene product from binding to β-2 microglobulin and interferes with its presence on the cell surface. On the other hand, the H63D polymorphism does affect protein binding and protein expression, but it is thought to affect the interaction of the *HFE* protein with the transferrin receptor (15). C282Y polymorphism is the most common polymorphism in hemochromatosis patients, and most affected individuals are homozygous for this polymorphism. However, the role of the H63D polymorphism in the pathophysiology of the disease remains unclear (16).

2. Objectives

Hemochromatosis meets most of the world health organization's criteria for performing an immense population screening. Since the frequency of the polymorphisms that predispose individuals to this disease may differ in each population, and there have been no reports on the northeastern population of Iran, the aim of this study was to assess whether polymorphisms in this gene could cause a predisposition to HCV infection. We compared the genotype and allele frequencies of H63D and C282Y polymorphisms in a group of HCV patients and healthy subjects in Mashhad, northeastern Iran.

3. Patients and Methods

3.1. Population Study

An archive of approximately 400 patients who were referred to the Imam Reza hepatology clinic over the last 15 years were reviewed by the authors, and each record was checked for a valid contact address. All procedures were carried out according to the principles of the institutional guidelines, and the study was approved by the ethics committee of Mashhad University of Medical Sciences. Written informed consent was obtained from all subjects prior to recruitment. Of those who had a valid address (N = 100), only 15 responded to the survey. In addition to these participants, 54 HCV patients who were referred to the Ghaem and Imam Reza hospitals from September 2012 to September 2013 were also recruited for our study. An additional 69 healthy individuals were recruited as controls. ELISA testing using commercial ELISA kits (Delaware, USA) was done to detect HBsAg-positive cases, who were then excluded. All participants were also checked for HIV infection. Ten milliliters of peripheral blood were collected in EDTA-containing collection tubes from each patient. All factors that could affect the risk of HCV infection, including drug and alcohol addiction, transfusions, and tattoos, were included in the final analysis when results were adjusted for confounders.

3.2. Genomic DNA Extraction and Polymorphism Genotyping

A DNA extraction kit (Genet Bio, Korea) was used to extract DNA, which was then stored at -20°C until the time of use. The polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) method was performed to genotype the HCV patients and the healthy controls, based on the study of Gharib et al. (17). The PCR process was performed for each location using a primer pair, as listed in Table 1. Each PCR reaction mixture (30 μl) contained 0.4 mM of each primer, 1.5 mM of $MgCl_2$, 0.4 mM of each deoxynucleotide triphosphate (dNTP), 0.5 U of Taq DNA polymerase (Genet Bio, Korea), and 40 ng of genomic DNA in 10X reaction buffer. PCR reactions were performed in a thermocycler machine (Applied Biosystems, USA). The PCR conditions are shown in Table 2. The PCR products of the *HFE* gene, 280 bp for exon 2 (His63Asp) and 390 bp for exon 4 (Cys282Tyr), were then observed by electrophoresis on 1.5% agarose gel (Genet Bio, Korea) stained by Green Viewer (Pars Tous, Iran). The amplification products were digested, using two restriction enzymes, RsaI (Fermentase, Germany) and BclI (Fermentase, Germany), in order to recognize the C282Y and H63D polymorphisms, respectively. The digested products were run on 3% agarose gel and stained with the Green Viewer staining method. The mutant allele has two sites for C282Y locus digestion, which causes the PCR products of 390 bp to be digested into three pieces: 250 bp, 111 bp, and 29 bp. The wild-type allele, which possesses one digestion site, is indicated by 250 bp and 140 bp bands. In this case, the presence of three 250 bp, 140 bp, and 111 bp bands was indicative of heterozygosity. For enzymatic digestion of the H63D locus, digestion of a 280 bp product into 138 bp and 70 bp fragments was indicative of the wild allele, and the existence of a band of 208 bp was indicative of a homozygous mutated allele with no enzymatic digestion site.

Table 1. Primer Sequences

Primer	Sequence
His63Asp (H63D) (forward)	5'-ACATGGTTAAGGCCTGTTGC-3'
His63Asp (H63D) (reverse)	5'-GCCACATCTGGCTTGAAATT-3'
Cys282Tyr (C282Y) (forward)	5'-TGGCAAGGGTAAACAGATCC-3'
His63Asp (H63D) (reverse)	5'-CTCAGGCACTCCTCTCAACC-3'

Table 2. PCR Cycle Conditions

Step	Temperature, °C	Duration	Repeats
Initial denaturation	94	2 min	1
Denaturation	94	1 min	35
Annealing	58	15 s	
Extension	72	30 s	
Final extension	72	7 min	1

3.3. Biochemical Assay

Two commercial ELISA kits (Pars Azmoon, Iran, and Pishtaz Teb, Iran) were used to analyze the levels of serum alanine aminotransferase (ALT) enzyme and serum ferritin, based on the manufacturer's instructions.

3.4. Statistical Analysis

Statistical analysis was conducted using SPSS version 21 (SPSS Inc., Chicago, IL, USA). For determination of normality, the data were assessed with the Kolmogorov-Smirnov test. The allele frequency and polymorphism distribution at two positions of the hemochromatosis gene, in HCV patients and healthy controls, were analyzed using the χ^2 test. PHASE software (v2.1) was used for haplotype and diplotype analyses. A $P < 0.05$ was considered significant.

4. Results

4.1. Demographics

Of a total of 69 HCV-infected patients enrolled in the current study, 61 (87.14%) were male and 8 (11.42%) were female. The control group was composed of 69 individuals, of which 22 (31.42%) were male and 47 (67.14%) were female. Statistical comparisons demonstrated significant differences between these two groups ($P < 0.05$). The average age of the patients affected by hepatitis C was 41.41 ± 9.87 years, which was not significantly different from the average age of 37.91 ± 12.53 years in the control group.

4.2. Biochemical Results

The serum ferritin and ALT levels in the patients and controls are shown in Table 3. Increased serum ferritin and ALT levels were observed in 43 (61.42%) and 20 (28.57%) patients and in 8 (11.42%) and 15 (24.42%) healthy controls, respectively. All participants in the present study were checked for HIV infection, and the results were negative for both the patients and the healthy controls.

Table 3. Demographic Parameters and Serology Results

Characteristic	Patients	Controls	P Value
Gender			
Male: Female	61: 8	22: 47	< 0.05
Age, y (mean ± SD)	41.41 ± 9.87	37.91 ± 12.53	0.08
Addiction, No. (%)	31 (44.28)	0	< 0.05
Alcohol, No. (%)	30 (42.85)	2 (2.85)	< 0.05
Transfusion, No. (%)	31 (44.28)	9 (12.85)	< 0.05
Tattoo, No. (%)	21 (30)	0	< 0.05
ALT, U.L-1 (mean ± SD)	33.93 ± 31.55	26.78 ± 22.92	0.28
Increased ALT[a], No. (%)	20 (28.57)	15 (24.42)	0.33
Ferritin, U.L-1 (mean ± SD)	325.57 ± 171.98	108.24 ± 58.05	< 0.05
Increased ferritin[b], No. (%)	43 (61.42)	8 (11.42)	< 0.05

[a] (> 41 U.L-1 for men and > 31 U.L-1 for women).
[b] (> 300 U.L-1 for men, > 200 U.L-1 for women, and > 100 U.Ll-1 for postmenopausal women).

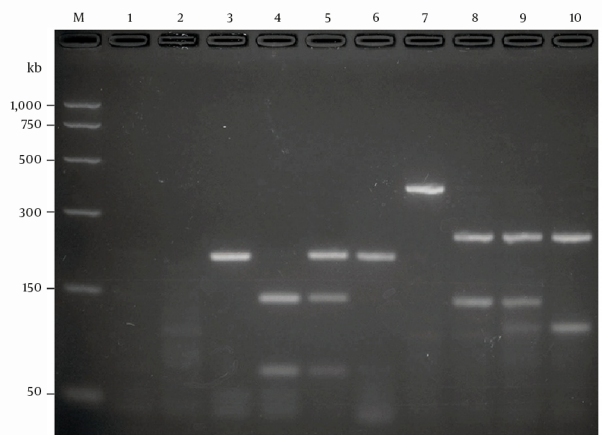
4.3. HFE Gene Polymorphism Analysis

The frequencies of the HH, HD, and DD genotypes were 86.15%, 4.61%, and 9.23% in the HCV patients and 49.23%, 38.46%, and 12.30% in the controls, respectively. The genotype distributions of the C282Y locus for the CC, CY, and YY genotypes were 84.61%, 10.76%, and 4.61% in the HCV patients and 64.61%, 0%, and 35.38% in the healthy controls, respectively. The gel electrophoresis results for the PCR-RFLP analysis of the wild type and mutant type positions of C282Y and H63D are shown in Figure 1.

H allele frequency was significantly higher in the HCV group than in the control group (88% vs. 68%; $P < 0.05$; OR = 3.53 [95% CI 1.84 - 6.79]). After adjusting for confounders, including transfusions, tattoos, and addiction (drugs and alcohol), which might influence the risk of HCV infection, there was still a significant difference between the cases and the controls (adjusted $P < 0.05$; OR = 4.35 [95% CI: 1.30 - 14.63]).

In a recessive model analysis of the H63D locus (HH vs. HD + DD), HH was significantly higher in the HCV patients (86.15%) than in the controls (49.23%; $P < 0.05$; OR = 6.42

Figure 1. Gel Electrophoresis of PCR-RFLP Analysis of Wild Type and Mutant type Positions of C282Y and H63D



Lane M, DNA size marker; lane 1, H63D negative control; lane 2, C282Y negative control; lane 3, uncut H63D fragment; lane 4, HH genotype; lane 5, HD genotype; lane 6, DD genotype; lane 7, uncut C282Y fragment; lane 8, CC genotype; lane 9, CY genotype; lane 10, YY genotype.

[95% CI 2.73 - 15.10]). This difference remained significant after adjustment for confounders (adjusted $P < 0.05$; OR = 6.42 [95% CI 1.51 - 27.33]).

Using the same analysis model for the C282Y locus (CC vs. CY + YY), CC was significantly higher in the HCV patients (84.61%) than in the controls (64.61%; $P < 0.05$; OR=3.01 [95% CI 1.29 - 7.01]), which remained significant after adjustment for confounders (adjusted $P < 0.05$; OR = 5.06 [95% CI 1.13 - 22.06]).

4.4. Haplotype and Diplotype Analyses

To perform haplotype and diplotype analyses using PHASE version 2.1, we assessed the frequency of haplotypes and diplotypes in the HCV patients and control subjects. The frequencies of haplotypes and diplotypes are listed in Table 5, which shows that the HC haplotype was more frequent in the HCV patients ($P < 0.05$; OR = 0.17 [95% CI 0.05 - 0.54]), while the HY haplotype was more frequent in the healthy controls ($P < 0.05$; OR = 0.33 [95% CI 0.16 - 0.67]). In addition, HCDC diplotype carriers were significantly more common among the control subjects than among the HCV patients ($P < 0.05$; OR = 0.18 [95% CI 0.05 - 0.65]).

5. Discussion

Polymorphisms in the *HFE* gene are a main cause of hepatic diseases. Two polymorphisms in the *HFE* gene result in a change of amino acid sites at codon 282 (Cys282Tyr) and codon 63 (His63Asp) (16). More than 90% of British patients with hereditary hepatic diseases are homozygous for at least one of these polymorphisms (18). Some reports have shown the relationship between accumulation of excess iron in the liver (19) and increased fibrosis in hepatic diseases associated with HCV (20-22). We demonstrated that the frequency of the C282Y polymorphism in HCV patients was significantly less than in healthy controls, and this result was opposite to that of other researchers (6-8). The frequency of the H63D polymorphism in HCV patients was also significantly less than in the healthy controls. Our findings did not support the results of the study conducted by Piperno et al. (19), in which it was claimed that there was no significant variation in the frequency of *HFE* gene polymorphisms between the case and control groups.

Although hepatic iron concentrations and transferrin saturation levels were not evaluated in the present study, the serum ferritin level has been used as a marker for hepatic iron storage in patients with chronic hepatitis (23). HCV patients usually have a high level of serum iron markers, which may be related to hepatic iron accumulation (24). Most researchers believe that *HFE* gene polymorphisms play an important role in iron accumulation and thus in the concurrent development of viral hepatitis. However, it is not clear whether increased iron indices is a phenomenon resulting from viral hepatitis, hepatic inflammation, and virus proliferation, or whether simultaneous *HFE* gene polymorphisms can affect hepatitis-induced hepatic diseases (25). Nonetheless, the effect of C282Y is attributed to iron accumulation (20). Our results may endorse the hypothesis that suggests that *HFE* gene polymorphisms affect the development of viral hepatitis or even hepatic diseases, regardless of the impact of iron overload. These results are probably related to the fact that the *HFE* gene encodes MHC-1 protein, which may have an impact on immune responses (6).

A significant difference was found between H63D heterozygosity among the HCV patients and the controls, which implies a mild effect of this polymorphism on hepatitis C disease. This is in contrast to the results obtained by Erhardt et al. (25). As a statistically significant difference was observed in the YY genotype frequency of the C282Y locus between the HCV patients and the healthy controls, it could be concluded that the heterozygous individuals for the C282Y polymorphism may not have been at higher risk of HCV infection. This result is in contrast with that of Erhardt's and Smith's studies (20, 25) on hepatitis C patients. The study performed by Smith et al. (20) reported that heterozygous hemochromatosis patients had more fibrosis related to chronic hepatitis C. Erhardt et al. (25) obtained similar results in a larger population. Based on the fact that the current study would not be capable of proving

Table 4. Genotype and Allele Frequencies in HCV Cases and Healthy Controls

locus	Controls No. (%)	HCV patients No. (%)	P Value Crude	OR Crude (95% C.I.)	P Value Adjusted[a]	OR Adjusted[a] (95% CI)
His63Asp (H63D)						
HH	32 (49.23)	56 (86.15)	< 0.05	6.42 (2.73 - 15.10)	0.012	6.42 (1.51 - 27.33)
HD/DD	33 (50.76)	9 (13.84)	< 0.05	6.42 (2.73 - 15.10)	0.012	6.42 (1.51 - 27.33)
DD	8 (12.30)	6 (9.23)	0.57	0.72 (0.24 - 2.22)	0.57	0.72 (0.24 - 2.22)
HD/HH	57 (87.69)	59 (90.76)	0.57	0.72 (0.24 - 2.22)	0.57	0.72 (0.24 - 2.22)
Allele frequency						
H	89 (68)	115 (88)	< 0.05	3.53 (1.84 - 6.79)	0.02	4.35 (1.30 - 14.63)
D	41 (32)	15 (12)	< 0.05	3.53 (1.84 - 6.79)	0.02	4.35 (1.30 - 14.63)
Cys282Tyr (C282Y)						
CC	42 (64.61)	55 (84.61)	< 0.05	3.01 (1.29 - 7.01)	0.03	5.06 (1.13 - 22.06)
CY/YY	23 (35.38)	10 (15.37)	< 0.05	3.01 (1.29 - 7.01)	0.03	5.06 (1.13 - 22.06)
YY	23 (35.38)	3 (4.61)	< 0.05	0.09 (0.02 - 0.31)	0.9	0
CC/CY	42 (64.61)	62 (95.37)	< 0.05	0.09 (0.02 - 0.31)	0.9	0
Allele frequency						
C	84 (64.61)	117 (90)	< 0.05	4.93 (2.51 - 9.69)	0.001	9.37 (2.5 - 35.3)
C	84 (64.61)	117 (90)	< 0.05	4.93 (2.51 - 9.69)	0.001	9.37 (2.5 - 35.3)

[a] Adjusted based on transfusions, tattoos, and addiction.

Table 5. Haplotype and Diplotype Frequencies in Cases and Controls

Locus	Controls, No. (%)	HCV Patients, No. (%)	P Value	OR	95% CI
Haplotypes					
DC	22 (15.7)	15 (10.7)	0.22	0.64	0.32 - 1.30
DY	17 (12.1)	1 (0.7)	< 0.05	0.05	0.007 - 0.39
HC	70 (50)	112 (80)	< 0.05	0.17	0.05 - 0.54
HY	31 (22.1)	12 (8.6)	< 0.05	0.33	0.16 - 0.67
Diplotypes					
HCHC	28 (40)	10 (18.2)	< 0.05	0.248	0.12 - 0.51
HYHY	10 (14.3)	10 (18.2)	< 0.05	5.67	1.19 - 26.90
HCDC	14 (20)	9 (16.4)	< 0.05	0.18	0.05 - 0.65
HYDY	11 (15.7)	11 (20)	< 0.05	12.86	1.61 - 102.6

the presence of any clinical relationship, and with regard to the findings of this review and the lower frequencies of the respective C282Y and H63D polymorphisms in HCV patients compared to healthy controls, similar investigations with larger numbers of HCV patients are recommended to provide stronger evidence of the association of *HFE* gene polymorphisms and the natural process of HCV.

The percentage of HCV-infected individuals who carried the C282Y polymorphism in this investigation (10%) was similar to that found in German (11.8%) (26) and French (11.4%) (27) studies, while it was higher than the estimations obtained from northern England (7.3%) (20), Italy (4% or 4.5%) (19, 28), and Brazil (4.5%) (29). The D allele frequency of the *HFE* H63D polymorphism was higher in our HCV patients compared with Italian (18.5%) (19) and Brazilian (12.9%) (29) patients.

The present study evaluated the frequency of *HFE* gene polymorphisms in normal samples from populations of northeastern Iran. The results showed that the allele frequencies for C282Y and H63D in our healthy subjects,

which may reflect the general population, were 35.38% and 32%, respectively. The respective frequencies of heterozygous C282Y polymorphisms in North American and southeastern European populations are reported as 9% and 1% - 3%, respectively (30). Other studies have reported the frequency of this polymorphism as 0% to 1.2% in different countries throughout the world (31-39).

The frequency of the H63D allele in our normal population was slightly higher than the rate reported in a neighboring country, Turkey (24.8%) (40). Other studies from different parts of the world have revealed an H63D allele prevalence ranging from 0.8% to 4.3% (31-35, 37, 39, 41, 42). Among cases with similar genotypes, males had higher levels of serum ferritin, which is in line with other studies and is probably due to menstrual blood loss in women.

The present data suggest that polymorphisms at the C282Y and H63D positions of the hemochromatosis gene are one of the risk factors for infection with hepatitis C. A larger number of HCV patients and healthy controls will be crucial in order to clearly illustrate the role of *HFE* gene polymorphisms. Although the present study may contribute to elucidating the role of genetic susceptibility in HCV infections, a better conception of the role of *HFE* gene polymorphisms and other factors related to the genetic background of HCV patients is vital for the expansion of novel preventative strategies.

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Footnotes

Authors' Contribution: Sina Gerayli: suggested the primary idea, assisted in performing laboratory tests, and wrote the primary manuscript; Alireza Pasdar: analyzed and interpreted the data, and revised the manuscript; Mohammad Taghi Shakeri: obtained funding for the study, and analyzed and interpreted the data; Samaneh Sepahi and Sina Rostami: assisted in performing laboratory tests; Mitra Ahadi and Seyed Mousalreza Hoseini: evaluated the HCV-infected participants, informed them about the disease, and helped them to better understand their infections; Zahra Meshkat: obtained funding for the study, helped in the conception and design of the study, and was the guarantor of integrity of the entire study.

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