Relationship Between Hemochromatosis Gene Mutations and Degree of Fibrosis in Liver Disease Associated with Chronic Hepatitis B and C

Kronik Hepatit B ve C ile İlişkinden Karaciğer Hastalığında Hemokromatozis Gen Mutasyonları ile Fibrozisin Derecesi Arasındaki İlişki

**ABSTRACT**

Objective: There are various factors that affect the degree of liver fibrosis in chronic viral hepatitis (CVH). Deposition of iron in the liver is one of these factors. Although hemochromatosis gene (HFE) mutation is determined as heterozygote, it is thought that it causes deposition of iron in the liver. However, the effect of the deposition of iron as a result of HFE gene mutation that leads to the progression of fibrosis in patients with CVH is still uncertain. The purpose of this study is to evaluate the association of HFE gene mutations with serum iron indices and degree of fibrosis in patients with CVH.

Material and Methods: The study enrolled 83 patients with chronic hepatitis B, 61 patients with chronic hepatitis C and 50 healthy controls. Fifty-two of the patients also had cirrhosis. AST, ALT, serum iron, ferritin and transferrin saturations were measured and the presence of HFE mutations were investigated for all individuals. Liver biopsy was performed to all patients. Histopathological findings in the liver were scored as necroinflammatory activity and fibrosis according to the Knodell scoring scale.

Results: The C282Y mutations could not be detected in any individuals. H63D heterozygous and homozygous mutations were found but they did not differ between patients (n=29, 20.1%) and controls (n=14, 28%). Presence of H63D mutations was not associated with serum iron indices or the degree of hepatic fibrosis.

Conclusion: Our results showed that H63D gene mutations do not have a significant role in the progression of fibrosis in CVH.

**Key Words:** Hemochromatosis; hepatitis B, chronic; hepatitis C, chronic; liver cirrhosis


**Anahtar Kelimeler:** Hemokromatozis; hepatit B, kronik; hepatit C, kronik; karaciğer sirozu


Mild to moderate iron overload is frequently observed in the liver of patients with chronic viral hepatitis (CVH). Increased amounts of iron in the liver may promote the progression of liver
disease by adding oxygen free radicals that increase oxidative stress.\textsuperscript{3,4} Iron overload is responsible for liver damage through the generation of reactive oxygen species leading to lipid peroxidation and alteration of the cellular membrane.\textsuperscript{5} Moreover, some studies suggest that iron excess in the liver may favor the persistence of viral infection and could have a negative influence on interferon response, and its removal might have beneficial effects by reducing serum aminotransferase levels.\textsuperscript{6-11}

The hemochromatosis gene (HFE), which is very frequent in whites, could have a role in the development of hepatic iron overload in CVH in these populations.\textsuperscript{2,12} The recent isolation of the hemochromatosis gene, a major histocompatibility complex class I-like gene now designated HFE, provides the opportunity of analyzing the role of HFE gene mutations on the development of iron overload in patients with CVH directly.\textsuperscript{13} Two missense mutations have been identified. One of them changes the 282 cysteine residue to tyrosine (C282Y) and it disrupts a b2-microglobulin binding site.\textsuperscript{13,14} The other mutation changes histidine at position 63 to aspartic acid (H63D).\textsuperscript{13-15} The causal role of the C282Y mutation in the development of iron overload is supported by the high frequency of C282Y homozygosity in hemochromatosis patients and by experimental data whereas the role of the H63D mutation is more controversial.\textsuperscript{13-17} The H63D mutation is frequent in normal controls but various observations suggest that it may have a role in hemochromatosis, but with a low penetrance.\textsuperscript{13,15,17}

Genetic variations in the HFE gene may act in at least 2 ways to influence development or progression of chronic liver disease: By leading to an increase in hepatic or total body iron levels or by modulating immune responses. The HFE gene mutations may also serve as markers of other HLA-associated genetic factors that influence inflammatory liver disease due to linkage disequilibrium.

In this study, we analyzed the frequency of both mutations in CVH patients and in healthy controls. In addition, we evaluated the association of the HFE gene mutations with degree of fibrosis, type of hepatitis and serum iron indices in patients with CVH.

**MATERIAL AND METHODS**

**PATIENTS**

We studied 144 patients (66 women, 78 men) with CVH; they had a mean (±SD) age of 48±13.6 years (range, 13-80 years). The diagnosis was based on persistent or fluctuating, high serum aminotransferase levels for more than 6 months, presence of hepatitis B surface antigen with hepatitis B virus DNA or hepatitis C antibodies and hepatitis C virus RNA in the serum, and chronic hepatitis with or without cirrhosis at liver biopsy. Eighty-three patients had chronic hepatitis B (CHB) and 61 had chronic hepatitis C (CHC). Fifty-two of the patients (18 women, 34 men) had cirrhosis, all in Child’s class A. We excluded patients with a history of alcohol consumption, blood transfusion or parenteral iron therapy.

The control group included 50 apparently healthy subjects whose mean age was comparable with that of the patients (mean±SD, 41±13.2 years; range, 19-75 years). They were spouses of patients, blood donors at their first donation, and members of the hospital medical and nursing staffs with normal liver function tests and normal iron indexes (serum iron: 85.7 μg/dL; range, 26-167 μg/dL; transferrin saturation: 25±10%; range, 7%-45%; serum ferritin: 63±49 μg/L; range, 15-288 μg/L). None of the controls were hepatitis B surface antigen-or hepatitis C virus antibody-positive. All patients and controls were of Turkey origin.

**METHODS**

Transferrin saturation and serum ferritin were measured using standard methods (upper normal values in our laboratories: serum iron, 167 μg/dL; transferrin saturation, 45%; serum ferritin, 160 μg/L in women and 350 μg/L in men). Liver sections were stained with standard stains for histological evaluation in all the patients. All specimens contained at least six portal tracts. The histological examinations were performed by an independent
observer with no knowledge of the results of chemical analyses or HFE genotyping. The Knodell score and histological activity index (HAI) were used to stage liver disease. Hepatic iron concentration could not be determined in patients because of technical reasons.

Analysis of HFE gene mutations was performed retrospectively in all the subjects. Genomic DNA was extracted from peripheral leukocytes. The two mutations were detected using the polymerase chain reaction, followed by restriction with Rsal for C282Y and Bcll for H63D mutations.13,18

Each patient gave informed consent to genotype analysis and liver biopsy. The study was approved by the Institute Ethics Committee.

Statistical analyses were performed by the parametric t test, One Way ANOVA test, and Pearson’s correlation coefficient; nonparametric Mann-Whitney U test and Kruskal-Wallis H test. Differences between groups of patients and correlations between different variables were obtained using the statistical package of SPSS 18. The p<0.05 was accepted statistically significant. In addition, while necessary, after Kruskal-Wallis H test, Mann-Whitney U test was applied to groups, and p<0.05/n (number of group) was considered as statistically significant in these groups.

RESULTS

Of 144 patients, 52 patients showed severe hepatic fibrosis (stage 4, cirrhosis). Serum iron was increased in 16 patients (11.1%), and serum ferritin and transferrin saturations were increased in 28 patients (19.4%) with CVH, while in no case in the control group.

A total of 79.9% (115/144) of the patients and 72% (36/50) of the controls had none of the 2 HFE genetic variations studied and were considered wild type. In addition, C282Y mutation was not found in any patients or controls. The single genetic variation found was H63D, which was present as the only variation in 20.1% (29/144, including 2 homozygotes) of patients and 28% (14/50, including 2 homozygotes) of the controls.

The prevalence of AST, ALT, serum iron, transferrin saturation and H63D mutations (18.1% in CHB vs. 22.9% in CHC; p=0.47) did not differ between patients with chronic hepatitis B and C. Only mean ages and serum ferritin levels were statistically different (p<0.05) in patients with CHB and CHC, respectively. Ferritin was increased in 10 patients (12%) with CHB while in 18 patients (29.5%) with CHC (p<0.05) (Table 1).

The demographic and laboratory data of the cases were summarized in Table 1. AST and ALT levels were adjusted according to Age and Fibrosis Score factors and then adjusted variables were analyzed by suitable tests in tables.

We stratified the data of our patients according to fibrosis because increased amounts of iron in the liver due to HFE may promote the progression of liver disease (Table 2). In the Table 2, single patient with fibrosis 0 was evaluated with patients with fibrosis 1.

All the serum iron indices except transferrin saturation and H63D mutations were not associated with degree of hepatic fibrosis. Number of the patients with increased transferrin saturation, mean age, serum AST and ALT levels were higher in patients with cirrhosis than the others. HAI also was found as high in patients with fibrosis 2 and 3.

We also stratified data of our patients according to patient sex because of women may have lower serum iron markers compared to men (Table 3).

Serum ALT levels and transferrin saturation rates were higher in men than in women. However, other serum iron indices, HAI, degree of fibrosis and presence of HD3D mutation were not statistically different. The number of patients with increased ferritin was identical in both groups although serum ferritin levels were found as high in men. In addition, the number of patients with increased iron and serum iron levels were found as high in men, but the difference was not statistically significant (p=0.08 and p=0.11, respectively).

In evaluation of the patients with Spearman’s correlation coefficient, significant correlations
were found between serum iron and serum ferritin levels ($r=0.39; p<0.001$), and transferrin saturation and serum ferritin level ($r=0.53; p<0.001$). Serum iron level also correlated with serum aspartate transaminase ($r=0.22; p<0.01$), alanine transaminase levels ($r=0.19; p<0.05$), and the patients’ age ($r=0.27; p=0.001$). HAI and degree of fibrosis only correlated with the patients’ age ($r=0.39$ and $r=0.43; p<0.001$, respectively), neither in those with nor in those without H63D mutations.

In addition, when we compared the patients divided according to the presence or absence of H63D mutations, we did not find any differences for age, patient sex, serum iron indices, AST and ALT levels, HAI or degree of fibrosis.

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**DISCUSSION**

Since the seminal work of Blumberg et al., iron has been recognized as a factor that may influence the severity and course of chronic viral hepatitis. In early work, it was noted that patients with chronic hepatitis B or C often had increased serum ferritin and/or transferrin saturation. This has been confirmed recently in the large data set from NHANES III comparing subjects with and without chronic hepatitis C as well as in the HALT-C cohort. In our study, higher levels of serum iron, ferritin and transferrin saturation were observed in patients with CVH compared to healthy controls ($p=0.007$, $p<0.001$ and $p=0.002$, respectively). None of the serum iron measures was related to liver fibrosis stage or necroinflammatory grade.
Although serum ferritin is well known to be a positive acute phase reactant, the increased frequency and degree of increase of serum ferritin level in chronic hepatitis C has been greater than for other types of chronic liver disease. Therefore, we found higher ferritin levels in patients with CHC compared to the patients with CHB. Iron deposits in the liver and abnormalities in serum iron biochemistry are frequently observed in patients with chronic liver diseases, but data for patients with hepatitis B virus (HBV) infection are scarce. Moreover, the role of HFE mutations in iron deposits in this condition remains unknown. Mar-
possible role of HFE mutations in the progression of CHC.\textsuperscript{28} However, while some studies have shown an association between mutated HFE genotypes and both iron overload and advanced fibrosis or cirrhosis, others have failed to document any significant involvement of HFE mutations in promoting hepatic iron accumulation and CHC evolution.\textsuperscript{24,29,30} A number of studies have addressed the role of the common HFE mutations in favoring hepatic iron accumulation during CHC, obtaining conflicting results.\textsuperscript{29,30} These discrepancies might be due to the failure to correct for confounding factors.\textsuperscript{30} Moreover, as pointed out by Tung et al., the association between HFE mutations and the severity of both iron accumulation and fibrosis is more evident in patients with compensated disease after controlling for the duration of infection, and disappears in patients with end-stage liver disease.\textsuperscript{31} Although most of the patients included in the present study have compensated CVH, we failed to see a contribution of HFE mutations in causing disease progression in patients with CHB or CHC. This might be ascribed to the predominance of the HFE H63D heterozygous variant among our patients, which affects iron homeostasis in CVH to a lower extent than the C282Y variant.\textsuperscript{32,33} Moreover, the presence of other, still poorly characterized, factors that interfere with the phenotypical expression of mutated HFE genes cannot be excluded.\textsuperscript{34} Furthermore, hepatic iron concentration could not be determined in our study that is the most important limitation of this study although Lin et al. showed that ferritin, iron and transferrin saturation were all excellent predictors for presence of hepatic iron overload in patients with CHC.\textsuperscript{35} In addition, we could not found C282Y mutations in any individ-

<table>
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<th>TABLE 3: The association of laboratory data and H63D mutations with sex.</th>
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<td><strong>Age (yr)</strong></td>
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<td>AST adj (U/L, mean ± SD)</td>
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<td>Iron (µg/dL, mean ± SD)</td>
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<td>Number (%) of patients with increased serum ferritin level</td>
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<td>T. Saturation (%), mean ± SD)</td>
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<td>Number (%) of patients with increased transferrin saturation</td>
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<td>Inflammation activity (HAI)</td>
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<td>Degree of fibrosis (Stage)</td>
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<td>H63D Mutations, number (%)</td>
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<td>Homozygous (H63D/H63D)</td>
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<td>Normal (Wt/Wt)</td>
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* Mann-Whitney U test.

HAI: Histological activity index.
uals. However C282Y mutation is very rare in Turkey, as it is known.\textsuperscript{36-40}

We also stratified the data of our patients according to patient sex because of women may have lower serum iron markers than men. In women, iron loss caused by menses and pregnancies is a major determinant of iron status, as shown by the significant differences in iron indexes between pre- and postmenopausal women. Iron loss may counterbalance the effect of HFE gene mutations on iron absorption, and can explain the lack of correlation between hepatic iron overload and HFE gene mutations.\textsuperscript{41} Accordingly, even in hereditary hemochromatosis, a significant number of homozygous women do not manifest the disease.\textsuperscript{42} We found that serum ALT levels and transferrin saturation rates were higher in men compared to women. However other serum iron indices, HAI, degree of fibrosis and presence of H63D mutation were not statistically different. The number of patients with increased ferritin was identical in both groups although serum ferritin levels were found as high in men. In addition, the number of patients with increased iron and serum iron levels were found as high in men, but the difference was not statistically important (\textit{p}=0.06 and \textit{p}=0.08, respectively). H63D mutation, HAI and fibrosis were not correlated with the iron indices in both sexes. On the other hand, Piperno et al. found a significant correlation between the H63D mutation and the presence of hepatic iron overload in the male patients.\textsuperscript{43} However in a recent study, Bonkovsky et al. found that although women had significantly lower levels of serum iron indices and alanine aminotransferase, H63D mutation was associated with increased iron loading in both sexes. In addition, there were no significant differences in the prevalence of HFE gene mutations among subjects with fibrosis (35.5\%) versus cirrhosis (32.9\%) in the study.\textsuperscript{24}

In the present study, we did not find any association between serum iron indices and degree of hepatic fibrosis or inflammation activity in patients with CVH. However, our study has shown that serum iron is associated with AST and ALT values, ferritin levels and mean age. The ferritin levels also showed correlation with transferrin saturation. That is, the biochemical injury of liver can be predicted by serum iron contents but the histological damage cannot. This is consistent with the finding that the decline in serum AST and ALT values after phlebotomy is not associated with a change in histological activity of inflammation or fibrosis.\textsuperscript{44} The mechanism by which iron accumulates in some patients with CVH is unclear. Whether this iron accumulation is the cause or the result of liver injury is unknown. Since serum iron index correlated significantly with the value of ALT, it was likely that the excess iron could be associated to its release from destroyed hepatocytes as a result of liver injury associated with CVH. This suggested that iron parameters in patients with CVH acted either as markers of the chronic inflammatory state or cytolytic liver activity, but did not directly reflect the progression of hepatic fibrosis.

Additionally, our study found that older age at biopsy was associated with severe hepatic fibrosis in patients with CVH. This is in accordance with previous studies showing that severity of HCV-related liver injury can be predicted by patient age.\textsuperscript{44,45}

Finally, we could not find C282Y mutations in any individuals and our results showed that H63D gene mutations do not have a significant role in the progression of fibrosis in CVH. In addition, we could not find any relation between H63D mutations and serum iron indices.

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**REFERENCES**

