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Original Article

Serum measures of iron status and *HFE* gene mutations in patients with hepatitis B virus infection

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Aim: We tested associations between *HFE* mutations and hepatitis B virus (HBV) infection. We also explored measures of total body iron status and their association with chronic HBV infection.

Methods: Serum measures of iron status and *HFE* mutations (C282Y, H63D, and S65C) were assessed in 344 Iranian patients with chronic HBV infection (214 asymptomatic carriers, 130 patients with chronic progressive liver disease [CPLD]) and 302 controls.

Results: Frequencies of *HFE* mutations did not differ between patients with chronic HBV infection and controls (C282Y: $P = 0.9$, H63D: $P = 0.8$, S65C: $P = 0.9$). By logistic regression, advanced hepatic fibrosis was associated with *HFE* H63D mutation (OR = 13.1, $P = 0.006$; 95% CI = 2.0–84.1). Higher levels of serum ferritin and transferrin saturation were observed in patients with CPLD than in healthy controls ($P = 0.0001$ and 0.01, respectively, adjusted for age and sex). None of the serum iron measures was related to liver fibrosis stage or necroinflammatory grade.

Conclusion: Serum iron measures are associated with chronic progressive hepatitis B. Carriage of *HFE* mutations is not associated with the presence of chronic HBV infection or values of serum iron measures in this population, although *HFE* H63D is associated with more advanced hepatic fibrosis.

Key words: fibrosis, hepatitis B, *HFE*, Iran, serum ferritin, transferrin saturation

INTRODUCTION

THERE IS GROWING evidence that increased body iron stores serve as a comorbid factor for development and/or progression of non-hemochromatotic (HHC) liver diseases. This subject has been extensively reviewed by Bonkovsky *et al.*[1–3] Recent evidence also indicates that the prevalence of *HFE* gene mutations in non-HHC, especially chronic hepatitis C (CHC),[4–7] non-alcoholic steatohepatitis (NASH),[8] and porphyria cutanea tarda (PCT)[9] is increased and that patients with CHC harboring especially the major C282Y mutation are more likely to progress to advanced hepatic fibrosis or cirrhosis.[5,10]

Blumberg *et al.* and Lustbader *et al.* were among the first to propose a relationship between body iron and outcome of hepatitis B and clearance of hepatitis B virus.[11,12] They found that hemodialysis patients with higher levels of serum ferritin were more likely to develop persistent chronic hepatitis B after acute HBV infection. In support of their results, a beneficial effect in response to interferon therapy was achieved by reduction of serum ferritin levels in individuals with chronic hepatitis B (CHB).[13,14]

We previously reported a higher frequency of the *HFE* C282Y mutation in a series of Iranian patients with chronic HBV infection compared to the controls.[15] No significant difference was found between patients with or without this mutation regarding serum ferritin levels. In the current study, we aimed: (i) to analyze the frequency of three *HFE* mutations associated with a phe-

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notype of iron overload (C282Y, H63D, and S65C) in a larger number of Iranian patients with chronic hepatitis B to understand whether *HFE* mutations are associated with the presence of chronic hepatitis B and/or progression of the disease; (ii) to investigate whether chronic hepatitis B is associated with increased serum measures of iron status; and (iii) to understand whether *HFE* mutations in chronic hepatitis B are associated with increased iron stores.

METHODS

Patients

A TOTAL OF 344 Iranian outpatients with chronic HBV infection (270 men, 74 women) who attended Tehran Hepatitis Center between June 2003 and August 2004 were enrolled consecutively in the study. All patients were invited to take part in the study. Informed consent was obtained from all participating individuals, and the study protocol was approved by the Ethics Committees of Shaheed Beheshti University of Tehran and The University of Connecticut Health Center (where genetic testing was performed). The diagnostic criteria for chronic HBV infection were seropositivity for hepatitis B surface antigen (HBsAg) for at least 6 months and lack of anti-hepatitis B surface antibody (HBsAb) using commercially available ELISA kits (Diasorin, Italy). One patient, who was coinfected with hepatitis C virus, was excluded from the study. All patients were seronegative for anti-HIV antibody. Concentrations of serum ferritin (RADIM M108 kit, Pomezia, Italy), serum iron, and transferrin (Lysis [AutoAnalyzer], Milan, Italy) were determined. Transferrin saturation with iron was calculated as (serum iron [μg/dL] × 100/[transferrin (g/L) × 25) × 5.558]).[16] Serum levels of aminotransferases (AST and ALT), total protein and albumin were also measured. Sera from all patients were tested for antismooth muscle antibody and were found negative. Serum ceruloplasmin and alpha-1 antitrypsin levels were normal. None of the patients consumed >2 drinks (30 mL) of alcohol per week.

Of the 344 HBV patients, 214 (162 men, 52 women) were considered to be asymptomatic carriers (ACs), based on persistently normal serum levels of ALT (5–40 U/L) (at least three determinations separated by at least 1 week), and negative serum HBeAg or positive HBeAb. The other 130 patients (108 men, 22 women) were considered to have chronic progressive liver disease (CPLD), manifested by elevated ALT levels, and by clinical, liver histological findings, or presence of HBeAg in the serum. Asymptomatic patients with less than 1.5 times normal persistent ALT elevation were diagnosed as CPLD, when they had either positive serum HBeAg or histological findings of hepatic necroinflammation. Serum HBV DNA (Cobas Amplicor HBV Monitor Test, v2.0, lower limit of detection: 200 copies/mL) was detected in patients with CPLD. Liver biopsies were carried out in 72/130 patients with CPLD. Liver biopsy slides were scored for necroinflammatory activity (histology activity index, HAI) and fibrosis stage using modified Knodell's scoring system.[17] All the liver biopsy specimens were interpreted by a single pathologist who did not know the group allocation of the patients or the results of *HFE* mutational or serum analyses. None of the ACs or patients with CPLD had a history of treatment with interferon, nucleoside analog, or phlebotomy prior to enrollment to the current study.

In addition, 304 suitable unrelated volunteer blood donors (272 men and 32 women) were enrolled as study controls. They presented to the Iranian Blood Transfusion Center of Tehran. None of them was HBsAg, HCV antibody or HIV antibody positive, nor had a history of liver disease. Only those who had normal serum ALTs were enrolled in the study. Control subjects who were positive for HBcAb ($n = 47$) were tested for serum HBsAb; all were positive.

Genotyping

Genomic DNA was extracted from whole blood using a DNA purification kit (Promega, Madison, WI, USA). Twenty-five ng of DNA was used for each of the genotyping assays.

HFE C282Y mutation analysis

The C282Y mutation was genotyped using the TaqMan 5′ nuclease assay with primers (600 nM each) $^{5'}$ATTGGGGATGGGACCTACCA$^{3'}$ and $^{5'}$TCACATACC CCAGATCACAATGAG$^{3'}$ together with 120 nM each of Cys-allele probe Fam-CCACCTGGcACGTATAT-MGB and Tyr-allele probe Vic-CCACCTGGtACGTATAT–MGB with ABI Universal 2x MasterMix in a total volume of 10 μL. PCR amplification using 96-well plates involved 42 cycles of 95°C for 15 s followed by 60°C for 60 s. Post-PCR fluorescence plate reads were carried out using an ABI Prism 7700 Sequence Detector (ABI, Foster City, CA, USA), and analyzed using ABI Sequence Detector v1.7 software.

HFE H63D and S65C mutation analyses

To screen subjects for the H63D and S65C mutations, fragments containing both mutation sites were PCR amplified using 250 nM of primers $^{5'}$GAAGCTTTGGGCTACGTG

Table 1 Characteristics of the study subjects

Characteristic	Healthy controls	Patients with HBV		
		Asymptomatic carriers	CPLD	Total
Number, total (M/F)	304 (272/32)	214 (162/52)	130 (108/22)	344 (270/74)
Mean age ± SD, y	41.7 ± 12	36.7 ± 12	38.1 ± 12	37.2 ± 13
Serum ALT (IU/L)	26.5 ± 10	27.9 ± 11.1	88.7 ± 54	51.3 ± 45
Serum AST (IU/L)	23.6 ± 7	26.9 ± 9	69.2 ± 38	43.0 ± 32
Serum TBR (mg/dL)	ND	1.47 ± 7.8	1.3 ± 0.9	1.4 ± 5.9
PT/INR, mean ± SD	ND	13.0 ± 0.3/ 1.0 ± 0.0	14.8 ± 12.0/ 1.1 ± 0.2	13.9 ± 8.6/ 1.08 ± 0.2

ND, not determined; PT, prothrombin time; TBR, total bilirubin.

GAT$^{3'}$ and $^{5'}$TTCTACTGGAAACCCATGGAGTTC$^{3'}$ in a Perkin Elmer 9600 Thermocycler. The PCR products were digested with Bcl I or Hinf I endonucleases (New England BioLabs, Ipswich, MA, USA) which distinguish the H63D and S65C mutations, respectively. A melting curve analysis was then performed in the presence of 50 µL of 10% DMSO, 20 mM NaCl, and 1X SYBR green dye. Samples were melted at 70–98 °C over 15 min and real time changes in SYBR green fluorescence were measured with the ABI Prism 7700 Sequencer Detector. Melting profiles were analyzed using Dissociation Curve Software v1.0. Repeat genotyping was routinely carried out for 10% of the samples for quality control purposes for all genotyping assays, with 100% concordance of results.

Data analysis

Multivariate logistic regression model was employed to find the association between a set of explanatory variables and the outcome measures. *HFE* mutations, serum ferritin, transferrin saturation, age and sex were chosen as the candidate variables for the model. Comparisons were performed among study groups including healthy controls, ACs and patients with CPLD.

All *P*-values are two-tailed. Statistical analyses were performed by using STATA version 9 (StataCorp LP, College Station, TX, USA) and Power and Precision Software 2.0.37 (Biostat Inc., Englewood, NJ, USA).

RESULTS

TABLE 1 SHOWS selected characteristics of the study subjects.

HFE mutations

The frequencies of *HFE* genotypes in patients and controls are reported in Table 2. Two HBV patients (0.58%) and two controls (0.68%) were heterozygous for the C282Y mutation. Sixty-three (18.5%) patients and 50 (17.1%) controls were H63D heterozygous, one (0.29%) patient and four (1.36%) controls were H63D homozygous, while one (0.29%) patient and one (0.34%) control were S65C heterozygous. Controlling for age and sex, none of the mutations was significantly more frequent in patients than in controls (C282Y: $P = 0.9$, H63D: $P = 0.8$, S65C: $P = 0.9$). Analysis of *HFE* mutations between ACs and patients with CPLD revealed no significant difference (C282Y: $P = 0.9$, H63D: $P = 0.9$, S65C: $P = 0.95$). For the one male control who was heterozygous for both S65C and H63D, the serum iron measures were within the normal limits.

Table 2 Distribution of *HFE* genotypes in study groups

C282Y genotype	H63D genotype	S65C genotype	Controls, *n* (%)	Total patients, *n* (%)	AC, *n* (%)	CPLD, *n* (%)
CC	HH	SS	235 (80.4%)	273 (80.2%)	173(80.8%)	100 (79.3%)
CC	HD	SS	50 (17.1%)	63 (18.5%)	40 (18.6%)	23 (18.2%)
CC	DD	SS	4 (1.36%)	1 (0.29%)	0	1 (0.79%)
CY	HH	SS	2 (0.68%)	2 (0.58%)	1 (0.46%)	1 (0.79%)
CC	HD	SC	1 (0.34%)	0	0	0
CC	HH	SC	0	1 (0.29%)	0	1 (0.79%)

Due to technical limitations *HFE* genotypes were not determined in four and 12 DNA samples among patients and controls, respectively.

Table 3 Summary of hepatic histopathology from patients with CPLD

		Sex		
		Male ($n = 61$)	Female ($n = 11$)	Total ($n = 72$)
Fibrosis stage	mean ± SD	1.8 ± 1.1	2.4 ± 1.7	1.9 ± 1.2
	median	2.0	2.0	2.0
Necroinflammatory grade	mean ± SD	5.1 ± 2.4	6.4 ± 3.0	5.3 ± 2.5
	median	4.0	5.0	4.0
Total score	mean ± SD	6.9 ± 3.0	8.7 ± 4.2	7.2 ± 3.3
	median	6.0	7.0	6.0

Biopsies of all subjects who underwent liver biopsies were interpreted by a single experienced pathologist who had no knowledge of the clinical or other laboratory features.

Serum measures of iron status

Adjusting for age and sex, we found significantly higher mean levels of ferritin and transferrin saturation (TS) in patients with CPLD than in controls ($P = 0.0001$, 0.01, respectively). Figure 1 summarizes the distributions and differences of mean levels of serum iron measures in healthy controls, ACs and patients with CPLD. Highly statistically significant differences in ferritin levels were observed among the study groups ($P = 0.0001$). The mean level of TS was higher in patients with CPLD (42%), compared with TS level in ACs (31.5%), however, the difference did not reach statistical significance ($P = 0.3$).

Measures of disease severity

Liver histology

A summary of histological characteristics of patients with CPLD who had undergone liver biopsy is given in Table 3.

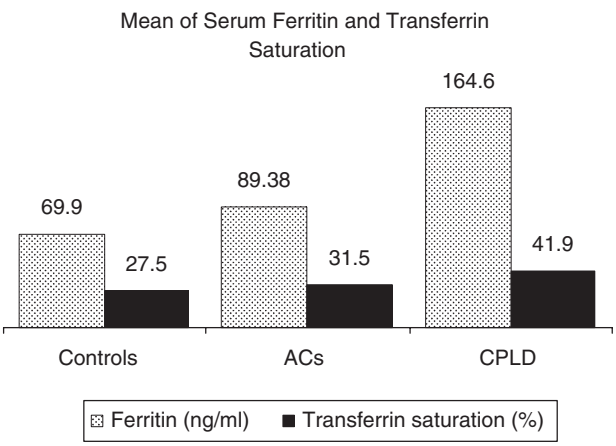

Figure 1 Distribution of serum measures of iron status in study groups. CPLD, chronic progressive liver disease.

Table 4 Distribution of H63D genotypes in different stages of hepatic fibrosis

Stage of fibrosis	(–/–) H63D	(+/–) H63D	Total
Mild	46	7	53
Moderate to severe	8	9	17
Total	54	16	70

We investigated the association between serum iron measures, frequencies of *HFE* mutations and the severity of liver injury in patients with CPLD whose pathology reports were available ($n = 72$). Among the independent variables considered (sex, age, ferritin, TS and *HFE* mutations), *HFE* H63D mutation was independently related to the liver fibrosis stage, evaluated as a dichotomous dependent variable (stages 0–2 = mild, stages 3–6 = moderate to severe) (OR = 13.1, $P = 0.006$; 95% CI = 2.0–84.1). None of the independent variables was statistically associated with the necroinflammatory grade (grade = 4 = mild, grade = 5–18 = moderate to severe). Serum levels of iron measures were not associated with stage or grade of liver histology ($P > 0.05$). Among 17 patients with moderate to severe hepatic fibrosis, nine were heterozygous for H63D. One of the patients with clinical, laboratory and imaging evidences of cirrhosis was homozygous for H63D. This patient did not undergo liver biopsy. Seven out of 53 CPLD patients with mild hepatic fibrosis were positive for H63D mutation. *HFE* mutations were not determined in two patients with mild fibrosis (Table 4).

DISCUSSION

TO OUR KNOWLEDGE, this is the largest study performed to date investigating the relationships among serum measures of iron status, the *HFE* gene

mutations associated with HHC, and CHB. The major results are that: (i) Iranian patients with chronic progressive hepatitis B have significantly increased levels of serum ferritin and TS compared with normal Iranian controls; (ii) serum ferritin and TS were not found to be associated with an increasing degree of severity of hepatic fibrosis or necroinflammation among patients with CPLD; and (iii) patients with chronic HBV infection do not have any difference in frequencies of *HFE* mutations (C282Y, H63D or S65C) compared with the controls. However, *HFE* H63D mutation is associated with more advanced liver fibrosis.

Baruch S. Blumberg first drew attention to the positive correlation between levels of serum iron, transferrin saturation, ferritin and persistence of HBV infection in patients on chronic hemodialysis.[11,12] Indeed, total body iron and hepatic iron (with or without *HFE* gene mutations) have been implicated in the pathogenesis and/or progression of several liver diseases, especially CHB, CHC, NASH, alcoholic liver disease, and PCT.[1–3,5–10] Reduction in serum ferritin by limited therapeutic phlebotomies (well short of iron depletion) significantly improved the likelihood of response of CHB to interferon alpha therapy.[13,14] In addition, therapy of CHB with lamivudine led to a significant decrease in serum ferritin,[18] prompting the investigators to suggest that sequential measurement of serum ferritin levels may be helpful in longitudinal evaluation of such patients.

Although a recent smaller study ($n = 75$) showed an increased prevalence of the C282Y mutation in Iranian subjects with chronic HBV infection (3/75, 4%) vs. normal controls (0/194, 0%),[15] the present study, involving nearly five times as many HBV patients, fails to support those earlier results. Thus, at least in patients with chronic HBV infection from central Iran, presence of HBV and/or increased serum measures of iron are not significantly related to the C282Y mutation. Since we found very low frequencies of C282Y mutation in our patients and also in controls, it could be argued that the numbers of patients and controls studied were too small to provide adequate power. However, based upon power calculations derived from the earlier results,[15] the much larger number of subjects studied here would be sufficient to demonstrate a difference ($\alpha = 0.05$, $\beta = 10\%$) if, in fact, one existed. Based upon the present results, the sample size to achieve an acceptable level of power would be very large and practically unfeasible.

In our study, the minor mutations (H63D, S65C) of *HFE* also associated with the iron overload phenotype were not over-represented in patients with chronic HBV infection. Others have reported similar results.[19,20] However, our findings show that *HFE* H63D contributes to fibrosis progression in chronic hepatitis B. The association of more advanced liver fibrosis with *HFE* mutations is in keeping with most, although not all, earlier studies in patients with chronic hepatitis C.[10,19,21] There was no evidence that carriage of *HFE* H63D mutation was associated with elevated serum iron measures.

Whether an *HFE* mutation is associated with more advanced hepatic fibrosis through an iron-independent mechanism is unknown. Mah *et al*. also demonstrated that H63D mutation was associated with the presence of liver fibrosis in chronic hepatitis B.[22] They did not evaluate hepatic iron content in the study subjects and could not demonstrate the penetrance of H63D mutation among those patients with hepatitis B. The product of the *HFE* gene is a major histocompatibility complex type I protein. As described elsewhere, subjects with *HFE* mutations represent several immunological differences compared to others without these mutations.[23,24] Hence, *HFE* mutations may lead to different host immune responses and affect cytokine production in patients with viral hepatitis. The presence of the H63D genetic variation in chronic hepatitis C is significantly associated with a higher likelihood of response to pegylated interferon plus ribavirin therapy.[21] Whether H63D mutation is in linkage disequilibrium or leads to gene–gene interactions with pro-fibrogenic or pro-inflammatory genes remain to be elucidated.

We observed a low allele frequency of C282Y mutation (0.34%) in our controls from Tehran compared with other Caucasian groups (6.8%), mostly from northern Europe and the USA.[25] Although the population of Iran is chiefly non-Celtic Caucasian, the low allele frequency of C282Y (0.0%–1.1%) shown here and in other studies on Iranian healthy subjects[15,26–28] points to the genetic diversity of Caucasian populations and supports the conclusion that this major mutation associated with HHC arose in those of Celtic origin.[29] Even among subjects homozygous for the major C282Y mutation, identified by mass screening studies, the penetrance of the iron overload phenotype is relatively low,[30] although it is much higher among relatives of known HHC subjects.[31]

The absence of association between increased serum iron measures and the frequencies of *HFE* mutations in our study suggests that in these patients iron overload can develop independently of the presence of the known *HFE* mutations. Based on the current study we cannot exclude that other genetic factors of iron overload may exist in these patients. The cause(s) of

increased serum ferritin and TS levels in patients with chronic viral hepatitis remain(s) uncertain. In addition to *HFE*, several other genes and their products have been shown to be important in iron metabolism and homeostasis, including DMT1, Dcytb1, ferroportin, hephaestin, transferrin receptors 1 and 2 and hepcidin.[32–35] Variations in expression of these genes are also important in modulating expression of the phenotype of iron overload.[36,37] With respect to liver diseases such as CHB, the expression of hepcidin, a bactericidal peptide made by hepatocytes, which regulates transport of iron across the upper small intestinal mucosa and release of iron from macrophages, seems likely to be of particular importance. Thus, liver diseases of diverse etiologies may downregulate hepatocytic production of hepcidin, leading to increased iron absorption, increased serum ferritin and increased total body iron stores. The increased iron may exacerbate hepatic (or other cell) injury, increase the risk of infections[38] and cancer,[39] and increase glucose intolerance and insulin resistance.[40,41] Thus, a vicious positive feedback loop of liver injury is established and maintained.

Although serum ferritin is known to be an acute phase reactant and thus might be expected to be increased in CHB or other active inflammatory liver diseases, we and others have found that serum levels of ferritin fall in such patients when iron is removed by phlebotomy[42,43] or by chelation.[13,14] Thus, we do not think hepatic inflammation *per se* can fully account for the increases in serum iron measures observed here and by many others. These findings support a role for iron depletion in patients with chronic hepatitis B, including those with elevated serum iron studies.

In conclusion, Iranian Caucasians with chronic HBV infection have evidence of increased levels of serum iron measures, but do not have frequencies of *HFE* mutations different from suitable controls. Whether variations in other genes involved in iron metabolism, or in immunologic or fibrogenic responses to chronic viral infection, are present in such subjects is a focus of ongoing research by our international research group.

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