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Iron overload and HFE gene mutations in Czech patients with chronic liver diseases

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Abstract. The aim of the study was to identify the prevalence of *HFE* gene mutations in Czech patients with chronic liver diseases and the influence of the mutations on iron status. The presence of *HFE* gene mutations (C282Y, H63D, and S65C) analyzed by the PCR-RFLP method, presence of cirrhosis, and serum iron indices were compared among 454 patients with different chronic liver diseases (51 with chronic hepatitis B, 122 with chronic hepatitis C, 218 with alcoholic liver disease, and 63 patients with hemochromatosis). Chronic liver diseases patients other than hemochromatics did not have an increased frequency of *HFE* gene mutations compared to controls. Although 33.3% of patients with hepatitis B, 43% of patients with hepatitis C, and 73.2% of patients with alcoholic liver disease had elevated transferrin saturation or serum ferritin levels, the presence of *HFE* gene mutations was not significantly associated with iron overload in these patients. Additionally, patients with cirrhosis did not have frequencies of *HFE* mutations different from those without cirrhosis. This study emphasizes the importance, not only of C282Y, but also of the H63D homozygous genetic constellation in Czech hemochromatosis patients. Our findings show that increased iron indices are common in chronic liver diseases but *HFE* mutations do not play an important role in the pathogenesis of chronic hepatitis B, chronic hepatitis C, and alcoholic liver disease.

Keywords: HFE gene, chronic hepatitis, alcoholic liver disease, hemochromatosis

1. Introduction

Although iron is essential for many biological processes such as erythrocyte development, DNA synthesis, and cellular respiration, excess iron is toxic via oxidative stress and production of reactive oxygen species that can lead to oxidation of lipids, nucleic acids, and proteins. Iron overload can thus cause serious damage to the liver by promoting hepatic fibrosis, cirrhosis, or even hepatocellular carcinoma [1,2]. *HFE*-linked hereditary hemochromatosis is an autosomal recessive disease characterized by iron overload. In 1996 a candidate gene for hemochromatosis named *HFE* was identified [3]. Inadequate function of HFE protein leads to excessive absorption of iron in the duodenum. Three common mutations in the *HFE* gene have been described in patients with hereditary hemochromatosis – major mutation C282Y (c.845G>A), and minor mutations H63D (c.187 C>G), and S65C (c.193A>T). Iron accumulation has been observed not only in hemochromatosis but also in many chronic liver diseases (CLD), although the exact mechanism by which iron is accumulated in the liver has not been elucidated yet. Some patients with alcoholic liver disease develop elevations in indices of iron stores [1,4,5] and the cumulative effects of ethanol and iron on liver cell damage, in patients with alcoholic liver disease, are topical issues. Abnormalities in serum iron biochemistry and liver iron deposits have been described in patients with chronic

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hepatitis B and C [1,2,6,7]. Several reports examining the relationship between *HFE* mutations and chronic liver diseases have been published; some have found evidence for an association between *HFE* mutations and iron overload, whereas others have not. The role of the heterozygous C282Y mutation in chronic liver diseases has received special attention; additionally, an increased prevalence of the H63D mutation has been found in some studies [1,2,7–9]. Recently, hepcidin has been recognized as the key regulator of iron metabolism. The role of this protein is examined not only in hemochromatosis but also in other chronic liver diseases with iron metabolism abnormalities such as viral hepatitis or alcoholic liver disease [10–15].

Controversial findings considering prevalence of *HFE* gene mutations were reported also for Czech CLD patients [16–18]. Therefore the aim of this study was to identify the prevalence of *HFE* gene mutations in Czech patients with chronic liver diseases (namely chronic hepatitis C and B, alcoholic liver disease, and hemochromatosis) and the influence of the mutations on iron status and course of the disease in these patients.

2. Subjects and methods

2.1. Subjects

The case-control study included 454 patients with chronic liver diseases (CLD) (275 male, 179 female), mean age of 55.4 years, ranging from 24 to 89 years. *HFE* genotyping was performed in 51 patients with chronic hepatitis B (HBV), 122 patients with chronic hepatitis C (HCV), 218 patients with alcoholic liver disease (ALD), and 63 patients with hereditary hemochromatosis (HHC). Patients living permanently in Bohemia were recruited at our out-patient department between 2000 and 2009. Chronic hepatitis C was diagnosed based on biochemical liver damage lasting more than 6 months and testing positive for the serum antibody to hepatitis C virus (anti-HCV) and HCV RNA presence in PCR tested serum. Patients with chronic hepatitis B tested positive for hepatitis B surface antigen (HBsAg) for at least 6 months. These patients were also tested for hepatitis B e antigen (HBeAg), antibodies to HBc (anti-HBc) and all tested positive for HBV DNA. All patients with chronic hepatitis B or C underwent a liver biopsy before treatment. The diagnosis of ALD was based on history of alcohol intake, presence of elevated serum AST (aspartate aminotransferase) or ALT (alanine aminotransferase) and GGT (gamma-glutamyltransferase) and sonographically observed fatty changes in the liver (liver steatosis). All subject in this group consumed more than 30 g alcohol per day. A group of subjects with clinical diagnosis of HHC was included in the study. The diagnosis of HHC was based on typical laboratory features (persistently raised iron indices, defined as serum ferritin $>$ 250 μg/L for males and serum ferritin $>$ 200 μg/L for females, transferrin saturation $>$ 45%) after excluding other causes of chronic liver injury e.g. alcohol abuse, viral hepatitis or drug toxicity. The diagnosis of cirrhosis was based on clinical, laboratory and ultrasonographic findings. Patients with cirrhosis did not present with symptoms of decompensation nor had had incidents of bleeding before recruitment. Alcoholics without cirrhosis had no clinical or laboratory signs of cirrhosis and ultrasonography showed no signs of portal hypertension. The prevalence of *HFE* gene mutations in the patients was compared with 481 controls from a previous study which was performed to determine the *HFE* gene mutation frequency in the general Czech population [19]. These samples represented randomly selected and anonymously tested dry blood spots (Guthrie cards) obtained from Czech National Newborn Screening Programme. The control group, which was used for comparing the iron status, consisted of 69 individuals who visited our out-patient department for reasons other than the above-mentioned diagnoses, usually for dyspeptic symptoms. They had no history of liver disease, their alcohol intake was below 30 g per day, were not diabetics and were not diagnosed as having viral hepatitis. Informed consent was obtained from all patients and the study was approved by the Ethics Committee of Third Faculty of Medicine and conducted in accordance with the Helsinki Convention.

2.2. Laboratory parameters

Biochemical testing (serum iron and ferritin concentrations, transferrin saturation, ALT and AST activities) was available in 46 patients with HBV, 108 patients with HCV, 193 ALD patients, 50 patients with HHC and 69 patients in the control group. However, complete biochemical data were not available for all non-hemochromatic patients; serum ferritin or transferrin saturation were available in 86 HCV, 36 HBV, and 190 ALD patients (Table 2). There were no differences in age, gender or *HFE* genotypes distribution between patients with known biochemical values and patients where biochemical values were not available within the particular diagnosis groups. Serum

Table 1
The frequency of *HFE* mutations and genotypes in patients with chronic liver diseases and controls

Diagnosis	HBV N = 51	HCV N = 122	ALD N = 218	HHC N = 63	controls N = 481
Alleles					
C282Y	2.9%	2.5%	2.3%	69.8%***†	3.4%
H63D	13.7%	10.7%	16.1%	23.0%*†	15.0%
S65C	3.9%	–	0.5%†	2.4%	1.2%
Genotypes					
C282Y/Wt	3 (5.9%)	6 (4.9%)	10 (4.6%)	12 (19.0%)***	33 (6.9%)
C282Y/C282Y	0 (0%)	0 (0%)	0 (0%)	38 (60.3%)***	0 (0%)
H63D/Wt	12 (23.5%)	22 (18.0%)	54 (24.8%)	13 (20.6%)	128 (26.6%)
H63D/H63D	1 (2.0%)	2 (1.6%)	8 (3.7%)	8 (12.7%)***	8 (1.7%)
S65C/Wt	4 (7.8%)	0 (0%)	2 (0.9%)	3 (4.8%)	12 (2.5%)
C282Y/H63D	1 (2.0%)	1 (0.8%)	0 (0%)	10 (15.9%)***	9 (1.9%)
C282Y/S65C	0 (0%)	0 (0%)	0 (0%)	1 (1.6%)	0 (0%)
H63D/S65C	1 (2.0%)	0 (0%)	2 (0.9%)	1 (1.6%)	2 (0.4%)
C282Y/C282Y or C282Y/Wt	3 (5.9%)	6 (4.9%)	10 (4.6%)	50 (79.4%)***	33 (6.9%)
H63D/H63D or H63D/Wt	13 (25.5%)	24 (19.7%)	62 (28.4%)	21 (33.3%)	136 (28.3%)
C282Y or H63D or S65C	18 (35.3%)	29 (23.8%)*	73 (33.5%)	62 (98.4%)***	170 (35.3%)
C282Y/C282Y or C282Y/H63D or H63D/H63D or C282Y/S65C	2 (3.9%)	3 (2.5%)	8 (3.7%)	57 (90.5%)***	17 (3.5%)

Wt – wild type. Significant differences: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ † Departure from Hardy-Weinberg equilibrium ($p < 0.05$, Chi-square test).

iron concentration, total iron binding capacity (TIBC), ALT and AST activities were measured using the VITROS method (Analysator Vitros Fusion 5.1), and serum ferritin concentration was measured using chemiluminescent immuno-analysis (Analyzator Immulite 2000, DPC). Detection of antigens and corresponding antibodies for hepatitis B and C was performed using ELISA (VITROS ECI, ORTHO Clinical Diagnostics). Transferrin saturation (TS – expressed in %) was calculated by dividing serum iron levels by total iron binding capacity (TIBC) and multiplying by 100.

2.3. DNA mutation analysis

C282Y, H63D, and S65C mutations of *HFE* gene were analyzed and detected in 454 patients. QiaAmp DNA Mini Kit spin columns (QIAGEN GmbH, Hilden, Germany) for DNA extraction from peripheral blood leukocytes were used and the extraction was performed according to the manufacturer's instructions. Examination of the *HFE* gene for the presence of mutations (C282Y, H63D, and S65C) used the PCR-RFLP method, as described previously [19].

2.4. Statistical analysis

Data are summarized as arithmetic mean ± SEM. The Chi-square test and Fisher's exact test were used to compare the frequency of genotypes between patients and controls and deviations from Hardy-Weinberg equilibrium (HWE) were calculated. Because many of the variables were not normally distributed, comparison of biochemical data between groups was performed using the Mann-Whitney test or the Kruskal-Wallis test with Dunn's Multiple Comparison test as appropriate. Statistical analysis was done using the GraphPad Prism program (GraphPad Software, Inc., version 5.00) and EpiInfo Software (version 6). The significance level was set at 0.05.

3. Results

3.1. Prevalence of HFE gene mutations in controls and patients with chronic liver diseases

No significant differences in the allele frequency of *HFE* gene mutations were observed between patients with chronic hepatitis B, hepatitis C or alcoholic liver disease and controls (general Czech population) (Table 1). The respective *HFE* genotypes were equally distributed between HBV patients and controls and ALD patients and controls. When comparing the presence of at least one of the *HFE* mutations between the

Table 2
The frequency of *HFE* mutations and genotypes in patients with chronic liver diseases according to the iron status

Diagnosis	HBV		HCV		ALD		Controls
Iron status	A	B	A	B	A	B	N = 481
	N = 12	N = 24	N = 37	N = 49	N = 139	N = 51	
Alleles							
C282Y %	8.3	2.1	2.7	3.1	1.4	4.9	3.4
H63D %	8.3	12.5	$16.2^{a,*}$	$7.1^{b,*}$	16.2	17.7	15.0
Genotypes							
C282Y/Wt %	16.7	4.2	5.4	6.1	2.9	9.8	6.9
C282Y/C282Y %	0	0	0	0	0	0	0
H63D/Wt %	16.7	16.7	$27.0^{a,*}$	$10.2^{b,*}$	23.7	31.4	26.6
H63D/H63D %	0	4.2	2.7	2.0	4.3	2.0	1.7
C282Y/H63D %	8.3	0	2.7	0	0	0	1.9
Wt/Wt %	75.0	75.0	67.6	$81.6^{b,*}$	69.1	56.9	64.7
presence of HFE mutation %	25.0	25.0	32.4	$18.4^{b,*}$	30.9	43.1	35.3

Iron status: A – serum ferritin >200/250 μg/L or transferrin saturation > 45%; B – serum ferritin <200/250 μg/L or transferrin saturation < 45%. a – group A vs. group B. b – particular group (A or B) vs. controls. Significant differences: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

HCV group of patients (23.8%) and the control group (35.3%) a significant difference was found ($p = 0.02$). As expected, the allele frequencies of C282Y and H63D mutations were significantly higher in patients with hemochromatosis compared to controls (general Czech population).

Severity of the disease was represented by the presence of cirrhosis and patients were divided according to this condition. When HBV, HCV, and ALD patients with cirrhosis were compared to corresponding patients without cirrhosis or controls (general Czech population) no significant changes were observed in allele or genotype frequency of *HFE* gene mutations.

3.2. Iron indices in patients with chronic liver diseases

When comparing iron indices of HCV, ALD, and HHC groups to controls, a significant increase was found in serum iron (HCV, ALD, HHC groups), serum ferritin (ALD and HHC groups), and transferrin saturation (ALD and HHC groups) (Supplementary Table 1). Serum iron levels above 26 μmol/L were detected in 22.7% of HBV patients, 39.2% of HCV patients and 37.2% of ALD patients. Transferrin saturation was increased to more than 45% in 28.1% of HBV patients, 27.7% of HCV patients, and 45.9% of ALD patients. Twenty percent of HBV patients, 35.7% of HCV patients, and 67.4% of ALD patients had ferritin concentrations above 250 μg/L in males and 200 μg/L in females, respectively. Elevated transferrin saturation or serum ferritin levels were present in 33.3% of patients with hepatitis B, 43.0% of patients with hepatitis C, and 73.2% of patients with alcoholic liver disease.

When the patients with hepatitis B and ALD were divided according to the presence of cirrhosis, no significant changes in their laboratory parameters were found. When the HCV group was divided based on the absence or presence of cirrhosis, cirrhotic patients had significant increases in serum iron levels (22.0 μmol/L vs. 27.1 μmol/L, $p = 0.02$), serum ferritin levels (232.8 μg/L vs. 320.2 μg/L, $p = 0.03$), transferrin saturation (33.9% vs. 45.4%, $p = 0.02$), and AST activity (0.9 μkat/L vs. 1.4 μkat/L, $p < 0.0001$).

3.3. The effect of respective HFE genotypes on iron status

The effect of particular *HFE* genotypes on iron indices in HBV, HCV, and ALD patients is summarized in Supplementary Table 2. Serum iron levels were significantly higher in H63D homozygotes ($p = 0.001$), but not elevated in other *HFE* genotypes when compared to *HFE* wildtypes. Serum ferritin or transferrin saturation levels were not increased in particular *HFE* genotypes when compared to patients with a wild type genetic constellation.

3.4. Prevalence of HFE gene mutations in patients with chronic liver diseases according to the iron status

HBV or ALD patients with elevated serum ferritin levels (cutoff: 200 μg/L for women and 250 μg/L for men) or increased transferrin saturation (cutoff = 45%)

did not carry *HFE* gene mutations significantly more often than corresponding patients with normal iron indices or controls (Table 2). In HCV patients with normal iron parameters, a significantly lower frequency of H63D mutation was detected than in corresponding HCV patients with elevated iron indices or with controls (7.1% vs. 16.2%, $p = 0.04$; 7.1% vs. 15.0%, $p = 0.03$, respectively). In addition, the prevalence of H63D heterozygotes was decreased in these patients compared to HCV patients with elevated iron indices or controls (10.2% vs. 27.0%, $p = 0.04$; 10.2% vs. 26.6% $p = 0.01$, respectively). The presence of all *HFE* gene mutations, in HCV patients with normal iron indices, was less frequent than in controls as well (18.4% vs. 35.3%, $p = 0.02$).

4. Discussion

The role of *HFE* gene mutations was investigated in chronic liver diseases in our study. It is speculated that *HFE* gene mutations, especially the C282Y mutation, even in heterozygous state, can contribute to iron loading and iron accumulation in the liver (via increased iron absorption due to the mutated HFE protein) and, through oxidative stress, may exacerbate existing liver disease [1,2,5,7]. This situation was documented in porphyria cutanea tarda (PCT) where the increased prevalence of the *HFE* gene mutation was shown in several studies [1,2,7,20,21]. However, this situation was not clearly evident in other chronic liver disease such as HBV, HCV, and ALD, where conflicting findings have been reported [1,2,7]. When we analyzed CLDs other than hemochromatosis, an increased prevalence of *HFE* gene mutations was not detected. Moreover, our study showed that *HFE* gene mutations were not associated with iron status in HBV patients. Although increased parameters of iron overload were observed in some patients, (33.3% with hepatitis B had elevated transferrin saturation or serum ferritin) there were no connections with *HFE* genotypes. In addition, even when patients were divided according to the severity of liver damage (i.e. presence of cirrhosis), there was no association with *HFE* gene mutations. Similar results were observed in some studies in HBV patients [6] whereas others have found that the presence of the H63D mutation tended to be associated with more advanced hepatic fibrosis or with progression toward liver cirrhosis in HBV patients [9,22]. Another study observed an increase in hepatic iron stores in C282Y heterozygotes and a correlation between the H63D mutation and iron overload only in male patients [23].

When patients with chronic hepatitis C were examined, 43% had elevated transferrin saturation or serum ferritin. HCV patients with cirrhosis had significantly higher iron indices (serum iron, serum ferritin, and transferrin saturation) in comparison to HCV patients without cirrhosis. However, HCV patients with cirrhosis showed no increase in the prevalence of *HFE* gene mutations compared to the general Czech population. When HCV patients were divided according to iron status, HCV patients with normal iron indices had a significantly lower prevalence of the H63D allele frequency and H63D heterozygotes compared to corresponding patients with elevated iron indices or the control population. It seems that in HCV patients, with elevated iron indices, the presence of the H63D mutation is increased, but only to the level found in the general Czech population. In addition, the presence of at least one *HFE* gene mutation was found to be significantly lower in HCV patients than in the control population; this difference was more pronounced in HCV patients with normal iron indices compared to controls. The role of the H63D mutation has so far been studied as a risk factor for iron accumulation rather than a protective factor [24–26]. Several studies have found an increased prevalence of *HFE* gene mutations in chronic hepatitis C with respect to the general population [27–29]. Some of the studies, which did not detect an increased prevalence of *HFE* mutations in HCV patients, found that (i) HCV patients carrying *HFE* mutations displayed higher iron indices when compared to wild-types (ii) being heterozygous for *HFE* mutations was an independent risk factor for progression of liver disease [16,30–33] or iii) did not find an association between iron accumulation and C282Y or H63D mutations at all [34–38]. Recently, the meta-analyses of 202 genetic association studies, involving a total of 66,263 cases and 226,515 controls, provided evidence of relationship between *HFE* genotypes and 31 disease endpoints. Subgroup analysis detected that homozygosity for the C282Y mutation was associated with hepatitis C (odds ratio 4.1) [20].

The association between increased serum iron concentrations and alcohol consumption led to evaluation of the role of *HFE* gene mutations in the pathogenesis of ALD. Both factors (iron and alcohol) cause oxidative stress and increases in reactive oxygen species (ROS) that are responsible for lipid peroxidation and liver cell damage [1,2,5]. However, the majority of studies found no relationship between *HFE* gene mutations and ALD

Table 3
Allele frequency in Czech patients with chronic liver diseases

Study	allele	ALD	ALD cirrhosis	ALD without cirrhosis	HBV	HCV
Putova et al., 2002 [18]	C282Y	–	7.9%	–	–	8.9%
	H63D	–	7.9%	–	–	8.9%
			N = 19			N = 23
Pucelikova et al., 2004 [17]	C282Y	2.4%	4.1%	0%	–	–
	H63D	17.9%	13.6%	23.5%	–	–
		N = 80	N = 40	N = 40		
Pacal et al., 2007 [16]	C282Y	–	–	–	3.9%	3.1%
	H63D	–	–	–	13.3%	9.8%
					N = 64	N = 143
Present study	C282Y	2.3%	2.5%	1.3%	2.9%	2.5%
	H63D	16.1%	15.9%	16.7%	13.7%	10.7%
		N = 218	N = 179	N = 39	N = 51	N = 122

or more advanced liver disease in ALD [4,39–41]. On the other hand, some studies observed a significantly increased frequency of H63D mutations in ALD patients compared to controls [8,42]. When patients with alcoholic liver disease were examined in our study, elevated parameters of iron status were observed compared to controls. The increase was not influenced by the presence of cirrhosis, i.e. no differences in iron indices were found in patients with and without cirrhosis. Despite biochemical evidence of iron overload in seventy-three percent patients with ALD, our study found similar allele frequencies of *HFE* gene mutations in ALD patients and controls. Even when patients with cirrhosis or elevated indices of iron status were studied separately, no increased prevalence of *HFE* mutations was observed. According to our study, it seems that *HFE* gene mutations are not associated with ALD.

Controversial results were found in previous Czech studies concerning CLD patients. One study found increased frequency of *HFE* mutations in patients with alcoholic cirrhosis and HCV [18], however the number of patients in this study was limited (23 patients with HCV and 19 with alcoholic cirrhosis). In contrast, other Czech studies including the present one did not find such associations [16,17], although one study showed that carriage of C282Y mutation tended to be associated with faster progression to decompensated liver disease in HCV patients [16] (Table 3).

As expected, our study demonstrated an increased prevalence of *HFE* gene mutations in Czech hemochromatosis patients. Sixty percent of our patients were homozygous for the C282Y mutation, which is less than what has been described in surrounding countries in Central Europe, where the frequency is over 80% [43, 44]. On the other hand, the prevalence of H63D homozygotes was 12.7%, which is higher than values reported for other European populations [25,26,43,44]. These findings emphasize the importance of the homozygous H63D mutation in Czech hemochromatosis patients.

It seems that molecules other than HFE are implicated in iron accumulation in CLD. Recently, the role of iron transport molecules such as ferroportin and transferrin receptors (TfR1, TfR2) together with the central regulator of iron metabolism - hepcidin - has been studied in HBV, HCV and ALD patients suggesting implication of these molecules in iron overload in these diseases [10–15,45,46].

We recognize that our study has some limitations. The biochemical data were not available for all patients as well as tissue biomarkers which could allow further exploration of the progression and severity of the liver disease. In addition, increase in serum ferritin could be related not only to iron overload but also to various types of inflammation which was not tested in our study.

In conclusion, our results showed that Czech patients with chronic liver diseases, such as chronic hepatitis B and C and alcoholic liver disease, do not have an increased prevalence of *HFE* gene mutations, although many of them displayed elevated serum iron parameters. In addition, patients with cirrhosis did not have frequencies of *HFE* mutations different from those without cirrhosis. Some studies documented the importance of *HFE* gene mutations in CLD, particularly with regard to HCV, where they were supposed to play a role in progression of liver disease while other studies, including this one, did not find such an association. Further research focused on molecules other than HFE is needed to elucidate the complex pathogenesis of iron accumulation in chronic liver diseases.

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Supplemental material

Supplementary data can be found at: http://www.lf3.cuni.cz/miranda2/export/sites/www.lf3.cuni.cz/cs/pracoviste/cph/publikace/supplementary-table1and2.pdf.

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