Lack of association of primary iron overload and common HFE gene mutations with liver cirrhosis in adult Indian population

Shalu Jain · Sarita Agarwal · Parag Tamhankar · Prashant Verma · Gourdas Choudhuri

Abstract
Aim To find out the association of common HFE mutations (viz., C282Y and H63D) with primary iron overload (PIL) in liver cirrhosis (CLD) patients of Indian origin.

Methods Polymerase chain reaction-restriction fragment length polymorphism method was used for screening C282Y and H63D mutation in 496 CLD patients (hepatitis B virus associated cirrhosis (HBVc)=74, hepatitis C virus associated cirrhosis (HCV)=50, alcoholic cirrhosis with hepatitis (ALcW)=38, alcoholic cirrhosis without hepatitis (ALc)=92, cryptogenic cirrhosis (CC)=242) and 502 healthy controls. Transferrin saturation of >45 or serum ferritin of >300 ng/mL (males)/>200 ng/mL (females) with normal total exogenous iron intake was suggestive of PIL. Histological liver iron grading was done by Perl’s Prussian blue stain.

Results Of 496 patients, 13 (2.6; 9 CC, 2 ALc, 1 HBVc, 1 ALcW) had PIL. However, only two (15.3) of 13 patients (1 CC and 1 HBVc) were positive for H63D heterozygous mutation. All the subjects were found to be C282Y wild type, except a single case of double heterozygous (C282Y/H63D) who however, did not have PIL. Overall frequency of H63D allele in patients and controls was not significantly different (5.95 and 4.58 respectively, p=0.17). A highly significant H63D allele frequency (p<0.005) was observed in HBVc (10.82) and ALcW (11.84) groups but they were not associated with PIL.

Conclusion The frequency of PIL, and the HFE gene mutation (C282Y) are both rare in Indian patients and explain why hemochromatosis is a rare cause of liver cirrhosis in India. A highly significant H63D allele frequency in HBV and alcohol-related cirrhosis suggest a possible predisposing role for liver fibrosis of this allele.

Keywords HFE mutations · Liver cirrhosis · Primary iron overload

Introduction
Iron is one of the key elements for cellular growth and development and is required for various essential processes in the human body. The controlled regulation of iron homeostasis is necessary to keep the body iron at a moderate level to avoid iron deficiency and iron overload [1]. There are several causes of iron overload like excess intake of dietary iron, chronic blood transfusion, chronic liver disease, porphyria cutanea tarda and dysmetabolic iron overload syndrome. Primary iron overload is generally due to hereditary hemochromatosis which is inherited either as autosomal recessive or as autosomal dominant manner. Hereditary hemochromatosis (HH) is characterized by defective iron metabolism which leads to iron deposition in various parenchymal organs notably the liver, heart, pancreas, pituitary gland, joints and skin with resultant end organ damage. Iron deposition accounts for various clinical complications such as arthritis, diabetes, cardiomyopathy, skin pigmentation, gonadal failure and liver cirrhosis (CLD) [2]. The genes identified to be responsible for
primary iron overload in HH are HFE, HJV, HAMP, TFR2 and SLC11A [3]. However, genes responsible for iron overload and not related to hereditary hemochromatosis are FTL, SOD and FTH1. Two missense mutations (C282Y and H63D) in HFE gene are found to be associated with HH phenotype [3]. The C282Y mutation, most frequent amongst Caucasians [3, 4] results from a G-to-A transition at nucleotide 845 of the HFE gene (845 G → A) that produces a substitution of cysteine for a tyrosine at amino acid position 282 (C282Y) in the protein product. Although C282Y mutations account for more than 80 of HH in North European populations [3], its frequency has been found to be very low (ranges between 0–0.5) [5], in Asians of the Indian subcontinent, African/Middle Eastern, and Australian populations.

In the H63D mutation, a C-to-G transversion at nucleotide 187 of the gene (187 C → G), results in a substitution of histidine for an aspartate at amino acid position 63 (H63D) in the HFE protein. The H63D mutation is widely distributed and has been identified in nearly all populations with the highest frequency of 30.4. Detected among Bosque population [5] H63D mutation correlates less well with HH, presumably contributing only in the condition of compound heterozygosity with the C282Y mutation [6, 7]. Few studies from India have found the presence of H63D mutation of the hemochromatosis gene, in 5–10 of normal controls, 8–9 of thalassemic and 7.6 of CLD patients [8–10]. Since liver cirrhosis by itself is also known to be associated with iron overload it may be expected that HFE mutations in such patients would worsen the iron overload. Iron deposition also is suspected of playing a synergistic role in the development of hepatic fibrosis, cirrhosis, and even hepatocellular carcinoma in association with chronic viral hepatitis, alcoholic liver disease, and non-alcoholic steatohepatitis [10]. The present study is an attempt to find out the prevalence of HFE gene mutation among patients with liver cirrhosis, compare them with controls and to delineate the role of common HFE mutations in iron overload.

Methods

Subjects

In the present study, 496 consecutive liver cirrhosis patients aged between 18–72 years (mean 42 [SD 11]) and 502 healthy controls were recruited from 2006 to 2008. Patients and controls were matched for age, sex and ethnic origin. The patients were clinically evaluated by the Department of Gastroenterology, Sanjay Gandhi Postgraduate Institute of Medical Sciences (SGPGIMS), Lucknow, India, which is a referral hospital in northern India. Control samples were collected from volunteer blood donors from the Department of Transfusion Medicine and blood donation camps organized by the blood bank of SGPGIMS, Lucknow. Institutional ethical clearance and written informed consent from all the patients and controls were obtained for the study.

Clinical and biochemical evaluation

The diagnosis of liver cirrhosis was based on clinical, biochemical and ultrasonographic features and confirmed by liver biopsy whenever possible. Histopathological assessment of liver iron load was done by the semiquantitative method of Scheuer et al. after staining with Perl's Prussian blue stain [11]. The etiology of cirrhosis were evaluated using appropriate serological (HBsAg, HBeAg, Anti-HBe IgM, Anti-HCV antibody, ANA, Anti-SMA, anti-AMA, ceruloplasmin, ferritin) and biochemical investigations (prothrombin time test, serum albumin, liver function tests, AFP). Alcoholic cirrhosis was diagnosed by history of prolonged heavy alcohol intake with AST: ALT ratio >2, and absence of other viral markers. Cryptogenic cirrhosis (CC) was diagnosed when a patient with liver cirrhosis had negative tests for HBV, HCV, autoimmune hepatitis, Wilson’s disease or alfa-1-antitrypsin deficiency, and did not consume alcohol [12]. After obtaining informed consent from all patients and healthy controls (voluntary blood donors), blood (2 mL) in EDTA vials, and (2 mL) in plain vials were drawn for molecular and serum iron studies respectively.

The concentrations of serum iron and total iron binding capacity (TIBC) were measured by ferrozine method and serum ferritin was estimated using enzyme immunoassay kit (Omega Diagnostics, UK) Transferrin saturation (TS) was calculated (Serum iron/TIBC × 100). Values considered normal were serum iron 20–150 microgm/dL; serum TIBC 250–450 microgm/dL, serum transferrin saturation 24–45, and serum ferritin 20–300 ng/mL. Biochemical iron overload was defined as transferrin saturation of more than 45 or serum ferritin more than 300 ng/mL in males or 200 ng/mL in females, on at least two separate occasions [5]. Primary iron overload was determined when increased iron intake from exogenous sources such as blood product transfusion, diet and iron formulations was ruled out.

Molecular analysis

Genomic DNA was isolated from the peripheral blood leukocytes by standard phenol chloroform method using whole blood. DNA fragments were amplified by polymerase chain reaction (PCR) and restriction enzyme analysis was performed for identification of C282Y and H63D mutations.
Primers used for C282Y mutation were Forward 5′- TGGCAAGGTAAACAGATCC 3′ and Reverse 5′-CTCACGTTCCTCTCAACC 3′. Primers used for H63D mutation were Forward 5′-ACATGTTAAGGCCGTGTTGC 3′ and Reverse 5′-GCCAATCTGGCTTGAAAAT 3′.

The PCR conditions were common for both mutations, i.e. 94°C for 10 min then 35 cycles of 94°C for 1 min, 62°C for 1 min and finally 72°C for 10 min. After amplification restriction enzyme digestion was carried out [6]. PCR product for C282Y mutation gives a band for 391 bp and an Rsa1 site is created in C282Y mutants. PCR product for H63D mutation gives a band at 208 bp. PCR product (10 μl) was digested with Mbo1 (10 U) and incubated overnight at 37°C. Normal allele give the bands at 250 bp and 140 bp after digestion whereas the mutant allele gives three bands at 250 bp, 110 bp and 30 bp. PCR product for H63D mutation gives a band at 208 bp. PCR product (10 μl) was digested with Mbo1 (10 U) and incubated overnight at 37°C. Normal allele gives two bands one at 138 bp and another at 70 bp whereas the mutant allele gives one band at 208 bp as the restriction site is lost in case of mutation. The frequency of the HFE alleles was calculated by gene counting. Statistical analysis of mutation prevalence in patient and control groups was performed using two-tailed Fisher’s exact test or chi-square test (p < 0.05 was considered as significant). Statistical analysis was performed using SPSS software version 12.

Results

Of the 496 patients, 92 had alcoholic cirrhosis, 38 had alcoholic hepatitis with alcoholic cirrhosis, 74 had HBV-related cirrhosis, 50 had HCV-related cirrhosis and 242 patients had cryptogenic cirrhosis. The prevalence of C282Y and H63D mutations in patients with cirrhosis and in the healthy control group are shown in Table 1. Neither the patients nor the controls were homozygous for C282Y mutation. However, heterozygous C282Y mutation was seen in one patient with CC along with heterozygous H63D mutation (compound heterozygous, C282Y/H63D). The patient had normal serum ferritin (56 ng/mL), iron (78 microgram/dL) and TIBC (210 microgram/dL) levels. Histological liver iron staining was normal (index 0).

Homozygous H63D mutation was seen in two patients; one with HBV related cirrhosis and other with CC. The serum ferritin, iron, TIBC and transferrin saturation levels for the HBV patient were 102.4 ng/mL, 88.12 mg/dL, 276.2 mg/dL, 31.9 and that for the CC patient were 92.12 ng/mL, 99.6 mg/dL, 312.3 mg/dL and 31.89. Histological liver iron staining showed normal findings (index 0).

Fifty-five patients were heterozygous for H63D mutation. Genotype (CG) frequency for H63D heterozygotes was 11.08 and the allele frequency for H63D was 5.95. Out of these 55 patients with heterozygous H63D mutation, 14 (18.9) had HBV-cirrhosis, 4 (8) had HCV-cirrhosis, 6 (6.5) had Alc, 9 (23.7) had AlcWand 22 (9.1) had CC (Table 2). None of these patients (except two discussed below) had biochemical iron overload and showed normal findings (Scheuer grade 0) on liver iron staining.

None of the controls were carriers of homozygous C282Y mutation, heterozygous C282Y mutation and homozygous H63D mutation and none had biochemical iron overload. However, 46 (9.16) controls were carriers of heterozygous H63D mutation. These carriers did not have biochemical iron overload. The genotype frequency for H63D heterozygous mutation (11.08) was similar in patients as compared to controls (9.16; p > 0.05). The allele frequency was similar in patients (5.95) as compared to controls (4.58; OR 1.31, 95 CI: 0.88–1.95, p = 0.17). When we studied the patients according to the etiology of the cirrhosis, differences were observed between allele frequency of two subgroup of patients i.e. HBV cirrhosis (10.8; OR 2.25, 95 CI: 1.39–4.59, p = 0.002) and alcoholic cirrhosis with hepatitis (11.8; OR 2.79, 95 CI: 1.31–5.96, p = 0.006) as compared to controls (4.6). In other subgroups allele frequency of H63D mutation was 4 for HCV cirrhosis (OR 0.86, 95 CI: 0.30–2.46, p = 0.79), 3.2 for Alc (OR 0.7, 95 CI: 0.29–1.66, p = 0.42) and 5 for CC (OR 1.08, 95 CI: 0.65–1.80, P = 0.74) as compared to controls (4.58; p = ns) (Table 2).

Of 496 patients with cirrhosis, 13 (2.6) were had primary biochemical iron overload (mean [SD] serum ferritin: 580.83 [234.16] ng/mL, mean transferrin saturation: 52.4 [8.2]). None of these patients had repeated blood transfusions or increased exogenous intake of iron. Among these thirteen patients, 9 (69.2) had CC, 2 (15.3) had AlcW and 1 (7.6) had HBV cirrhosis and 1 had AlcW. However, only two (15.3) of the 13 patients carried the H63D mutation.

Liver biopsy and iron staining was possible in 88 cases of CLD. Iron staining was done on all the liver biopsy specimens. This included the thirteen cases with biochemical iron overload and the rest (75) did not have biochemical iron overload (28 cases of alcoholic hepatitis, 12 cases of HBV overload).
C282Y mutation is the most common one described in patients with hemochromatosis [3]. C282Y mutation is the commonest one described in patients with hemochromatosis [3]. C282Y mutation hence was 0.05. The patient was a 54-year-old male with CC with no sign of primary iron overload. This may be due to low penetrance of iron storage disease in the Indian population [8, 10, 15]. This country, and underscores the genetic differences between populations. The presence of compound heterozygote in Indian population may be due to some admixture with European allele in the past. In contrast to C282Y mutation, H63D was more common in most of studies from India [5, 8, 18, 19]. The overall allele frequency of H63D mutation was 5.26 (4.58 in controls and 5.95 in patients) which is quite similar to the previously reported studies by Panigrahi et al. and Milman et al. [17, 20] but lesser than the others [8, 13, 25]. This difference is probably due to the larger sample size of our study as compared to others. Although the prevalence of HFE mutation in cirrhosis patients did not differ from that of controls in our study, the H63D mutation was significantly increased in two subgroups of cirrhotics: those due to HBV infection and those due to excess alcohol consumption. The prevalence of H63D mutation in HBV related cirrhosis patients was 10.8 compared to 4.58 in controls ($p < 0.005$). The similar high frequency (11.84) was noted in alcoholic cirrhosis (OR: 2.79, 95 CI: 1.31–5.96, $p = 0.006$). The exact reason for the high frequency of H63D heterozygosity in patients with HBV and alcohol-related liver cirrhosis is not clear. It is possible that mutation in this gene predisposes to development of cirrhosis in the presence of another cause of chronic inflammation of the liver such as chronic hepatitis B infection or alcohol. Several earlier studies have shown that H63D heterozygosity is associated with an increased risk for liver fibrosis and cirrhosis [19, 21, 22]. It may therefore be of interest to study whether patients with chronic hepatitis B or chronic heavy alcohol consumption who harbour the H63D mutation are at greater risk of progression to cirrhosis than their counterparts who do not have this mutated allele. Few authors did not however find this relationship in their studies [21–24]. There was no difference in HFE gene mutation between patients with HCV cirrhosis and controls in our study. This might be due to the small sample size within the group. This finding is in line with other studies [19, 22, 26].

Discussion

We have analyzed the serum parameters and HFE gene mutations (C282Y and H63D) in different subgroups of cirrhosis patients and controls to find a relationship between iron overload and HFE mutation in Indian population. Our findings show that biochemical iron-overload was seen in only 13 (2.6) of liver cirrhosis patients, only 2 of whom had a mutation of the HFE gene. None of these patients however had evidence of hemochromatosis. The genetic mutation and biochemical iron overload state did not therefore seem to be related in the majority (11 patients). Primary hemochromatosis appears rare in Indian patients, as we failed to find any in 496 consecutive patients with liver cirrhosis.

The second important finding of our study was the low frequency of the mutated C282Y allele in Indian subjects. Most of the earlier data, except a couple of studies [13, 14] showed complete absence of C282Y mutation in Indian population [8, 10, 15–17]. However, we have observed a single case of C282Y/H63D compound heterozygote for the first time in India. The resultant allele frequency for C282Y mutation hence was 0.05. The patient was a 54-year-old male with CC with no sign of primary iron overload. This may be due to low penetrance of iron storage disease in compound heterozygotes [3]. C282Y mutation is the commonest one described in patients with hemochromatosis in people of European descent. Its very low frequency in Indian subjects may explain the paucity of this disease in this country, and underscores the genetic differences between populations. The presence of compound heterozygote in Indian population may be due to some admixture with European allele in the past.

In contrast to C282Y mutation, H63D was more common in most of studies from India [5, 8, 18, 19]. The overall allele frequency of H63D mutation was 5.26 (4.58 in controls and 5.95 in patients) which is quite similar to the previously reported studies by Panigrahi et al. and Milman et al. [17, 20] but lesser than the others [8, 13, 25]. This difference is probably due to the larger sample size of our study as compared to others.

The exact reason for the high frequency of H63D heterozygosity in patients with HBV and alcohol-related liver cirrhosis is not clear. It is possible that mutation in this gene predisposes to development of cirrhosis in the presence of another cause of chronic inflammation of the liver such as chronic hepatitis B infection or alcohol. Several earlier studies have shown that H63D heterozygosity is associated with an increased risk for liver fibrosis and cirrhosis [19, 21, 22]. It may therefore be of interest to study whether patients with chronic hepatitis B or chronic heavy alcohol consumption who harbour the H63D mutation are at greater risk of progression to cirrhosis than their counterparts who do not have this mutated allele. Few authors did not however find this relationship in their studies [21–24].

There was no difference in HFE gene mutation between patients with HCV cirrhosis and controls in our study. This might be due to the small sample size within the group. This finding is in line with other studies [19, 22, 26].

### Table 2: Prevalence and allele frequency of H63D mutation in different subgroups of patients and controls

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>H63D</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Homozygotes (+/+)</td>
<td>Heterozygotes (+/−)</td>
<td>Wild type (−/−)</td>
<td>Allele frequency</td>
<td>$p$-value</td>
</tr>
<tr>
<td>HBVc (HBV related cirrhosis)</td>
<td>1 (1.3)</td>
<td>14 (18.9)</td>
<td>59 (79.7)</td>
<td>10.81 (16/148)</td>
<td>0.002</td>
</tr>
<tr>
<td>HCVc (HCV related cirrhosis)</td>
<td>0 (0)</td>
<td>4 (8.0)</td>
<td>46 (92.0)</td>
<td>4.0 (4/100)</td>
<td>0.79</td>
</tr>
<tr>
<td>Alc (alcoholic cirrhosis without hepatitis)</td>
<td>0 (0)</td>
<td>6 (6.5)</td>
<td>86 (93.5)</td>
<td>3.2 (6/184)</td>
<td>0.42</td>
</tr>
<tr>
<td>ALCW (alcoholic cirrhosis with hepatitis)</td>
<td>0 (0)</td>
<td>9 (23.7)</td>
<td>29 (76.3)</td>
<td>11.84 (9/76)</td>
<td>0.006</td>
</tr>
<tr>
<td>CRe (cryptogenic cirrhosis)</td>
<td>1 (0.4)</td>
<td>22 (9.1)</td>
<td>218 (90.0)</td>
<td>4.97 (24/482)</td>
<td>0.74</td>
</tr>
<tr>
<td>Control</td>
<td>0 (0)</td>
<td>46 (9.2)</td>
<td>456 (90.8)</td>
<td>4.58 (46/1004)</td>
<td>−</td>
</tr>
</tbody>
</table>

HBVc: HBV related cirrhosis; HCVc: HCV related cirrhosis; Alc: alcoholic cirrhosis without hepatitis; ALCW: alcoholic cirrhosis with hepatitis; CRe: cryptogenic cirrhosis.
Liver is the primary target organ in hemochromatosis and the major site of initial iron deposition. Both serum ferritin and transferrin saturation can be increased in liver diseases such as hepatitis B and C. In most of our patients, abnormalities of iron metabolism are independent of HFE mutation. In the present study only 2.6% of liver disease patients showed characteristics of primary iron overload. Amongst them only 2 H63D heterozygotes mutant were found, ignoring any role of HFE gene mutation in causing iron overload in liver cirrhosis patients of India.

Acknowledgement The Authors are thankful to DBT-New Delhi for providing financial support and SGPIMS for providing infrastructure facility to conduct the study. The Authors are thankful to Department of Biotechnology, Ministry of Science and Technology (Government of India) for providing financial support, by project No. BT/PR4205/Med/12/167/2003 granted to Prof. Sarita Agarwal.

References