Prevalence of HFE mutations and relation to serum iron status in patients with chronic hepatitis C and patients with nonalcoholic fatty liver disease in Taiwan

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INTRODUCTION

Hereditary hemochromatosis is one of the most common inherited diseases among Caucasians. It occurs with a frequency of 1:200 to 1:400 and a carrier rate approaching 1:100. Hereditary hemochromatosis is an autosomal recessive condition in which excessive iron is absorbed by the intestine. Iron accumulation in many organs causes the clinical manifestations including heart failure, diabetes, and liver cirrhosis. Early diagnosis has been difficult because the tests based on measurement of serum transferrin saturation and serum ferritin concentration not only gave many false positive results but do not always identify patients in the early stage of iron accumulation. The excessive iron can be removed by venesection. Although venesection is a simple, effective and safe therapy, much of the organ damage is irreversible when hereditary hemochromatosis is diagnosed. Because the major clinical presentations are often non-specific, appropriate screening tests are important.

The gene responsible for hereditary hemochromatosis was identified and designated as HFE in 1996. The two recognized recessively-inherited missense mutations of the HFE gene result in amino acid substitutions at position 282 (cysteine to tyrosine, C282Y) and at position 63 (histidine to aspartic acid, H63D). A total of 64-100% patients of Western population with hereditary hemochromatosis were C282Y homozygotes. In individuals of the northern European descent, the frequency of C282Y homozygote was about 0.5%. The allele frequencies for C282Y mutation and H63D mutation were 5-10% and 6-30%, respectively. In Taiwan, a very low frequency of C282Y mutation in the general population was reported. The C282Y homozygote may have higher serum and hepatic iron levels than controls, but levels are only mildly elevated. The clinical significance of the H63D mutation still remains undetermined. It seems to be associated with hereditary hemochromatosis only when inherited together with the C282Y mutation (compound C282Y/H63D heterozygote).

Mild to moderate hepatic iron overload is common in patients with chronic hepatitis C (CHC) and in patients with nonalcoholic fatty liver disease (NAFLD). Iron-induced oxidative stress may play an important role in the...
exacerbation of liver cell injury\textsuperscript{[19,20]}. The HFE mutation may also be associated with elevated markers of iron store in the two disorders. This study was performed to assess the prevalence of the two mutations, C282Y and H63D of HFE gene, in healthy subjects, patients with CHC, and patients with NAFLD in Taiwan. We also checked the serum iron markers and explored the contribution of the HFE mutation on serum iron stores in patients with CHC and patients with NAFLD.

**MATERIALS AND METHODS**

**Healthy subjects and patients**

C282Y and H63D mutations of HFE gene were analyzed in 125 healthy subjects, 29 patients with CHC, and 33 patients with NAFLD. Local healthy subjects with normal liver function test, no history of liver disease, other severe chronic disease, heavy drinking (ethanol consumption >20 g/d), anemia or iron overload were collected from the health checkup at our hospital in 2003. The patients with CHC or NAFLD were collected at our out-patient department from 2002 to 2003. CHC was diagnosed by biochemical liver damage for more than 6 mo and positivity of serum antibody to hepatitis C virus (HCV). Patients with secondary causes of iron overload were excluded, including heavy drinking, ribavirin therapy, and multiple transfusions. Co-infection with HBV was also excluded. The diagnosis of NAFLD was made on the basis of the presence of elevated serum AST or ALT and fatty change of liver by sonography. No subject consumed alcohol more than 20 g/d. They were negative for hepatitis B surface antigen and HCV antibody. Their serum levels of ceruloplasmin were within normal range. Serological tests for autoimmune hepatitis (anti-nuclear antibody, anti-smooth muscle antibody) and for primary biliary cirrhosis (anti-mitochondrial antibody) were negative.

**Serological evaluation**

The serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined using an Olympus 5000 analyzer. The upper limit of normal for ALT is 34 U/L. HCV antibody was tested by a commercially available ELISA (AxSYM, Abbott Diagnostic Corporation, USA). Total iron status of each patient was evaluated using biochemical tests. Serum iron (normal range, 60-160 μg/dL) was measured by the colorimetry and ferritin (normal range: 18-274 ng/mL) was measured by a commercially available ELISA (AxSYM, Abbott Diagnostic Corporation). Transferrin saturation was calculated as (the serum iron divided by the total iron binding capacity (TIBC)) ×100%. The increased serum iron store was defined by transferrin saturation >50% and/or ferritin >upper normal limit.

**Mutational analysis of HFE gene**

We used the method of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) to analyze the HFE mutations. Genomic DNA was isolated from whole peripheral blood using the High Pure Viral Nuclear Kit (Roche Diagnosis Corporation, USA). The DNA fragments of HFE gene were amplified by PCR using the following primers\textsuperscript{[5]}: 5'-TGGCAAGGTAAACAGATCC and 5'-CTCAAGCCTCCTCATAACC for C282Y; and 5'-ACATGGTAAAGGCCGTGTCG and 5'-GCCACATCTGGCITGAATTT for H63D. Restriction fragment length analysis was then performed by digesting the PCR products with RsaI for C282Y and BclI for H63D\textsuperscript{[21]}. The presence of C282Y and H63D mutations in the two disorders were studied by digesting the PCR products of HFE gene with RsaI and BclI. The DNA fragments of HFE gene were amplified by PCR using the following primers\textsuperscript{[5]}: 5'-TGGCAAGGTAAACAGATCC and 5'-CTCAAGCCTCCTCATAACC for C282Y; and 5'-ACATGGTAAAGGCCGTGTCG and 5'-GCCACATCTGGCITGAATTT for H63D. Restriction fragment length analysis was then performed by digesting the PCR products with RsaI for C282Y and BclI for H63D\textsuperscript{[21]}.

**Statistical analysis**

Data are summarized as mean±SD. Categorical variables were compared with the χ\(^2\) test or Fisher’s exact test as required. Comparison between groups was performed using the unpaired t-test as appropriate.

**RESULTS**

The demographic and laboratory data of subjects are summarized in Table 1. CHC patients were older than healthy subjects (60.83±8.55 years vs 44.62±11.19 years, \(P<0.01\)) and NAFLD patients were not (49.48±14.58 years vs 44.62±11.19 years, \(P = 0.082\)). There was no significant difference in the male to female ratio between healthy subjects (44/81) and CHC (12/17) or NAFLD (16/17) group.

All of the healthy subjects and patients were free from C282Y mutation. Four healthy subjects (one man and three women) were heterozygotes of H63D, two CHC patients (one man and one woman) and one NAFLD patient (woman) were heterozygotes of H63D. The prevalence of H63D mutation was 4/125 (3.20%) in healthy subjects, 2/29 (6.90%) in CHC group, and 1/33 (3.03%) in NAFLD.
The healthy subjects showed no significant difference in the prevalence of H63D mutation as compared with the CHC or NAFLD group (Table 2).

Table 2: Statistical analysis of H63D mutation between healthy subjects and CHC, and healthy subjects and NAFLD

|                      | Healthy subjects:CHC | Healthy subjects:NAFLD |
|----------------------|----------------------|------------------------|
| Male                 | 1/44:1/12            | 1/44:0/16              |
| Female               | 3/81:1/17            | 3/81:1/17              |
| Total                | 4/125:2/29           | 4/125:1/33             |
| \(P\)                | 0.386                | 0.999                  |

Table 3: Statistical analysis of increased serum iron stores between male and female patients with CHC or NAFLD

|                      | CHC                | NAFLD               |
|----------------------|--------------------|---------------------|
| Male:female          | 4/126:17           | 8/164:17            |
| \(P\)                | 0.913              | 0.114               |

Table 4: Statistical analysis of HFE mutation between patients with increased serum iron store and those without in CHC or NAFLD group

|                      | CHC         | NAFLD         |
|----------------------|-------------|---------------|
| With:without         | 1/10:1/19   | 0/121:21     |
| \(P\)                | 0.999       | 0.999         |

DISCUSSION

A population study of global prevalence in HFE mutation showed that there were nine H63D heterozygotes in one Asian population of 242 cases and no other HFE genotypes were found[1]. The H63D allele frequency was 1.9% (9/484). Similar results were reported in a Japanese population[2]. All of the 151 healthy volunteers were free from C282Y mutation and only 8 subjects were H63D heterozygotes. The prevalence of chromosomes with H63D was 2.6% (8/302). We firstly found no C282Y mutation and an incidence of 1.6% (4/250) for H63D allele in 125 healthy subjects in Taiwan. The results were similar to that in other Asian area. No mutations of C282Y were found probably because of the limited number of subjects studied. The very low frequency of HFE mutation in the Asians, especially C282Y, reflects the ethnic differences in the prevalence of hereditary hemochromatosis from Caucasians.

Similar prevalence of HFE mutations was found between controls and patients with CHC[22-25]. Our findings were in lines with these studies. However, the equal distribution of HFE mutations does not allow the conclusion that HFE mutations play no or only a minor role in patients with CHC. It should depend on the contribution of HFE mutations on the iron stores. Either the prevalence of C282Y mutation[18-20] or combined C282Y and H63D mutations[27] was increased in nonalcoholic steatohepatitis (NASH) population. Because there was no pathological diagnosis of steatohepatitis in our patients, the patient group in our study is called NAFLD, instead of NASH. Our study showed no significant difference in the frequency of HFE mutations between healthy subjects and NAFLD patients. In another study of NAFLD, the prevalence of the two HFE mutations also was not significantly increased in NAFLD patients and matched those of the general population. These findings gave us a hint that HFE mutation may play a role in the progression of NAFLD.

Elevations in serum iron, ferritin, and transferrin saturation are common in patients with CHC or NASH, as are mild increases in hepatic iron concentration. In CHC, 40-46% of patients had elevated serum iron, ferritin, or transferrin saturation level[14,18], and 10-36% of patients had increased hepatic iron concentration[15,19]. In NASH, 58% of patients had elevated serum iron indices and in some cases increased hepatic iron stores[60]. In our study, we found that 34.48% and 36.36% of patients had increased serum iron stores in CHC and NAFLD group, respectively. Normal or only mildly increased amounts of iron in the liver can be damaging because iron increases oxidative stress[19,20]. It appears that iron overload may play a role in pathogenesis of some chronic liver diseases, especially when iron is combined with other hepatotoxic factors such as virus, free fatty acid, and alcohol[3]. In addition to the effect of production of oxidative stress, the iron may enhance the rates of viral replication and mutation as well as cause impairment of the host immunity[20]. However, the iron can be released from damaged hepatocytes in inflammatory conditions, and it has not been clear whether the iron accumulation is the cause or result of liver injury.

Our data demonstrated no significant difference in the prevalence of HFE mutations between the subgroup of HCV or NAFLD patients with increased serum iron store and the subgroup without. The findings suggested that HFE mutations played no or only a minor role in contribution to serum iron overload in patients with CHC or NAFLD in Taiwan. In addition to the reason of low prevalence of HFE mutations in Taiwan, the fact that only H63D heterozygote was found in our study may explain the results. The H63D heterozygosity usually does not lead to iron overload. Only in conjunction with the heterozygous C282Y mutation (compound C282Y/H63D heterozygote) has the H63D mutation been associated with an increased risk of iron overload[3].

There were many reports suggesting that the C282Y mutation plays a role in the hepatic iron accumulation and favors the progression of CHC[23-25]. However, the relationship
between HFE mutations, iron stores, and NASH still remains as a controversial area. Previous studies have found conflicting results. George et al. found that the C282Y mutation was responsible for most of the mild iron overload in NASH and had a significant association with hepatic damage in these patients. Bonkovsky et al., also found that C282Y heterozygotes were more likely to have advanced fibrosis. These authors did not adjust for potential confounders such as age, body mass index, and diabetes. After adjustment for these confounders, Chitturi et al., reported in NASH and Bugianesi et al., reported in NAFLD patients that iron burden and HFE mutations did not contribute significantly to hepatic fibrosis. Therefore, we believe that continued exploration of the link between hepatic iron, HFE mutation, and NAFLD patients is warranted.

In conclusion, HFE mutations are infrequent in Taiwan. The prevalence of HFE mutations associated with hereditary hemochromatosis is not increased in the patients with CHC or NAFLD. The HFE mutations may not contribute to iron accumulation in the CHC or NAFLD group even when serum iron overload is observed in more than one-third of these patients in Taiwan.

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