C282Y Mutation and Hepatic Iron Status in Hepatitis C and Cryptogenic Cirrhosis

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Background.—Increased iron deposition in liver is seen in both primary and secondary hemochromatosis. However, it is not uncommon to see significant iron deposition in a liver biopsy, explant, or autopsy specimen without any significant clinical risk factor. Because of the discovery of the candidate gene (HFE) for hereditary hemochromatosis, we may now be able to screen high-risk patient populations for the abnormal mutation (C282Y).

Materials and Methods.—In this study we analyzed the livers of 50 transplant patients with a diagnosis of either hepatitis C cirrhosis or cryptogenic cirrhosis for the prevalence of the more common C282Y mutation of the HFE gene and correlated the findings to hepatic iron concentration.

Results.—Of the 26 cases of hepatitis C cirrhosis, 3 were found to be heterozygous for the C282Y mutation. Of the 22 cases of cryptogenic cirrhosis, 1 was found to be heterozygous for the C282Y mutation. Stainable iron was increased in hepatitis C cirrhosis (76.9%) as compared to cryptogenic cirrhosis (50%) (P = .05). Of the 3 heterozygotes with hepatitis C cirrhosis, 2 showed hepatic iron concentrations of 3+ and 4+, and 1 showed 1+.

Conclusions.—We conclude that patients with hepatitis C have an increased tendency to accumulate iron in the liver, and mutations in the HFE gene play a minor role in hepatic accumulation of iron in these patients.

(Arch Pathol Lab Med. 2000;124:1632–1635)

von Recklinghausen coined the term hemochromatosis to describe an autosomal-recessive condition associated with overabsorption of iron and its accumulation in solid organ parenchyma, particularly the liver, pancreas, and heart.1 Feder et al2 cloned a candidate gene responsible for hereditary hemochromatosis (HH) linked to the HLA-A1 locus on the short arm of chromosome 6 and named it HFE. A single G·A missense mutation in HFE, resulting in a cysteine to tyrosine substitution (C282Y), causes HH. This mutation is found in 64% to 91% of North American patients with HH.2–4 Hereditary hemochromatosis is a common disorder affecting 1 in 300 individuals.5,6 Overall, 83% of HH patients in the United States are homozygous for the C282Y mutation.2 A very small number of patients may have another mutation, H63D,3 however its role in the etiology of HH remains controversial.

Since suspect individuals can be screened for the abnormal mutation (C282Y), attention has been directed toward analyzing the association of this mutation to other known iron-overload conditions. Heavy iron deposition is commonly seen in cirrhosis regardless of etiology.7 It has already been shown that 44% of patients with porphyria cutanea tarda, a disease associated with iron overload, exhibit a mutation in at least 1 allele of the HFE gene.8 It is not known how the mutations on the HFE gene contribute to the pathogenesis of iron overload in porphyria cutanea tarda. Hepatic iron overload unrelated to HH also occurs in conditions such as alcoholic liver disease, hepatitis C, and cryptogenic cirrhosis.

Several studies have also suggested an association between hepatitis C and HH.9 Patients with chronic hepatitis frequently show an increase in serum iron indices and hepatic iron deposition.10–13 These patients who have high liver iron content respond poorly to interferon therapy.14,15 High iron content may also facilitate viral multiplication, may worsen cirrhosis, and may increase the risk of developing hepatocellular carcinoma.16,17 Based on liver iron content, HLA genotyping, and high iron indices, up to 10% of patients with hepatitis B or C would fall in the category of genetic hemochromatosis.10,18 Although these tests cannot identify the patients with heterozygous mutations of the HFE gene, polymerase chain reaction (PCR) analysis can readily be used to identify them. Explanted livers from patients undergoing transplantation can serve as models for determining the association between heavy iron deposition and the mutations associated with the HFE gene.

We therefore set out to study the presence of the HFE gene mutation in patients with hepatitis C and cryptogenic cirrhosis who underwent liver transplantation. In cases for which no cause for the development of cirrhosis could be determined, the diagnosis of cryptogenic cirrhosis was based on clinical data. The frequency of the more common of the 2 known mutations of the HFE gene (C282Y) was determined in 50 consecutive patients who underwent orthotopic liver transplantation for liver failure secondary to cryptogenic or hepatitis C cirrhosis. We then correlated the findings with the hepatic iron concentration in archival material of explanted livers (paraffin blocks). The blocks...
were numbered 1 through 50 and a blind analysis was carried out.

MATERIALS AND METHODS

DNA Extraction and Amplification

Two to 4, 4-μm-thick sections from archived paraffin blocks were cut for each case. DNA extraction was carried out using a standard, commercially available silica column method (QiAamp tissue kit, Qiagen, Valencia, Calif). The purified DNA was eluted with 60 μL of 0.01 mol/L Tris–hydrochloride (pH 8.0). All DNA solutions were kept at 4°C until PCR was performed.

DNA was amplified using sequence-specific primers. The PCR reaction mixture contained 50 mmol/L potassium chloride; 4 mmol/L magnesium chloride; 10 mmol/L Tris–hydrochloride, pH 8.3; 0.2 mmol/L of each of the deoxynucleoside triphosphates; and 0.3 units of AmpliTaq gold DNA polymerase (Perkin-Elmer, Norwalk, Conn). Primers for the wild type (nucleotide position 845G) and C282Y mutant (nucleotide position 845A) were those described by Smillie.19 Primers for the internal control, human growth hormone, were developed in our laboratory from the published sequence of the human growth hormone gene20 (forward primer 5'-TGG CTT CCC AAC CAT TCC CTT A and reverse primer 5'-ATT TCC AGC GGG GGA AAG TCA). The resulting PCR product is 150 base pairs in length. The PCR cycling conditions were as follows: 94°C for 9 minutes (hot start), followed by 30 cycles of 94°C for 45 seconds/66°C for 45 seconds, with a final extension at 66°C for 10 minutes. Polymerase chain reaction products were analyzed by electrophoresis on a 2% agarose gel. The DNA bands were stained with ethidium bromide and visualized on a Vistra Fluorimager SI.

Iron Concentration

The histologic sections of all 50 cases were reviewed and assessed for stainable iron on Perl Prussian blue stain. A semiquantitative method for assessing iron stains was adapted from Scheuer et al21 as follows: 1+, staining present in less than 25% of hepatocytes; 2+, staining present in 25% to 75% of hepatocytes; 3+, staining present in more than 75% of hepatocytes; and 4+, heavy staining present in almost all hepatocytes and also in the biliary duct epithelium.

In cases in which some variability in the intensity of the iron staining was observed, grading was done according to the more intensely stained areas. The reviewers (M.H. and P.L.) did not know the status of HFE mutation while assessing the iron stains.

RESULTS

A total of 50 samples were used for this study. Two samples could not be amplified owing to degradation of the DNA. A total of 4 heterozygous mutations were identified (8.3%) (Figure 1). Of the 48 samples thus analyzed, 26 were categorized as cirrhosis due to hepatitis C and 22 were cryptogenic cirrhosis. Three of the 4 mutations identified were observed among the 26 (11.5%) hepatitis C samples, and only 1 was observed among the 22 (4.6%) cryptogenic cirrhosis specimens (Table 1).

Stainable iron was evaluated in all of the 48 samples and graded semiquantitatively from negative, 1+, 2+, 3+, and 4+, according to the method described by Scheuer et al.21 The results of iron staining are shown in Table 2.

Of the 26 patients with hepatitis C, 6 did not show any iron, 7 had 1+, 4 had 2+, 5 had 3+, and 4 had a 4+ staining pattern for iron. Of the 22 patients with cryptogenic cirrhosis, 11 were negative for iron, 5 had 1+, none had 2+, 5 had 3+, and only 1 had 4+ staining. Stainable iron was increased in hepatitis C cirrhosis (76.9%) as compared to cryptogenic cirrhosis (50%) ($\chi^2, P = .0520$; Figure 2, Table 2).

Of the 3 heterozygotes with hepatitis C, 2 cases showed iron staining patterns of 3+ and 4+, and 1 showed only 1+. The results of the gene mutation and iron concentration studies are summarized in Table 3.

COMMENT

Iron deposition in liver and its role in modulating viral hepatitis have been well known for about 15 years.22 Stainable iron is seen commonly in liver biopsy specimens of patients with viral hepatitis, and an association exists between increasing severity of chronic viral hepatitis, fibrosis, and iron deposition.23 It has also been suggested that the distribution of stainable iron in the liver is useful in predicting the likelihood that chronic hepatitis C will respond to interferon alfa therapy.24-26 The presence of cirrhosis and the degree of iron deposition correlated inversely with response to interferon alfa.25 However, the cause and mechanism leading to increased iron deposition in chronic viral hepatitis remains unknown. It has been postulated that altered iron metabolism may be related to the effect of the virus itself.12 Also, not all patients with viral hepatitis accumulate hepatic iron. Other factors, including coexistence of HH and associated mutations, may play a role in contributing to the increased incidence of iron deposition in these patients. Previously, association of iron overload and cirrhosis was investigated by phenotypic markers, such as serum and hepatic iron content. These

Figure 1. Agarose gel electrophoresis of C282Y mutation analysis by sequence-specific polymerase chain reaction. Lane 1, 100 base pairs (bp) DNA marker; Lanes 2 and 3, patient 1 (Pt 1), who is heterozygous for the HFE gene; Lanes 4 and 5, patient 2 (Pt 2), who is homozygous normal for the HFE gene; and Lanes 2 through 5, 150-bp band, human growth hormone, internal control (IC).

Table 1. Results of Mutation Analysis*

<table>
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<th>Mutation Negative</th>
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<tr>
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<tr>
<td>Cryptogenic cirrhosis</td>
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* $\chi^2 P$ value = .371 (not significant).

Table 2. Results of Stainable Iron*

<table>
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<th>Etiology</th>
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<tr>
<td>Cryptogenic cirrhosis</td>
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* $\chi^2 P$ value = .0520 (borderline significant).
measurements are inadequate, as they do not identify mutations in the HFE gene, which may additionally contribute to iron deposition in liver.

In this study, mutation analysis was used to analyze the association of hepatitis C and cryptogenic cirrhosis with hepatic iron concentration. This allows distinction between HH-associated increased hepatic iron concentration and other causes of iron overload. It also allowed us to analyze the association of heterozygotes in hepatitis C–induced cirrhosis versus cryptogenic cirrhosis.

Patients with cirrhosis undergoing liver transplantation by definition have a more severe form of cirrhosis and liver dysfunction. The percentage of patients with hepatitis C carrying the heterozygous C282Y mutation in this study (11.5%) was higher than data published for hepatitis C patients without need for transplantation (England, 7.3%; Italy, 4% and 5.4%; and Brazil, 4.5%). Other studies of patients with hepatitis C from Austria (11.3%), Germany (11.8%), and France (11.4%) revealed a frequency similar to that of our study. This difference may indicate a genuine geographic or racial variation of genetic expression of the C282Y mutation. It may also indicate a higher frequency and penetrance of the heterozygous mutation in patients with more severe cirrhosis necessitating transplantation. It is possible that the presence of a mutation in the HFE gene may be an adverse factor in the progression of cirrhosis.

Of the patients in our study with hepatitis C, 88.5% did not carry the mutation; however, 76.9% of patients showed increased hepatic iron content. In a study by Hezode et al, 88.6% of the patients with hepatitis C failed to show the C282Y mutation of HFE, similar to our finding. However, only 42.2% of their patients showed increased hepatic iron load. The increased hepatic iron load (76.9%) in our study is most likely due to the selection of patients with end-stage liver disease. Our data show that in most patients with hepatitis C, abnormalities of iron metabolism are independent of HFE mutation. Out of a total of 4 mutations identified in our study, 3 (75%) showed 3+ or greater iron deposition. One case showed only 1+ iron deposition. In the study by Kazemi-Shirazi et al, hepatic iron load was not increased in 4 of 5 C282Y homozygotes with hepatitis C infection. The fact that all homozygotes for the C282Y mutation will not develop full expression of hemochromatosis has been well documented. It is therefore hardly surprising that one of the heterozygotes in our study did not reveal increased hepatic iron even at the end stage of liver disease (cirrhosis). Other factors contributing to iron overload in HH are host immune responses. Similarly, several studies have shown that a qualitative or quantitative defect in the immune response, impairment of function of iron-loaded antigen-presenting cells, inhibition of cloning efficiency of Th1 and cytotoxic lymphocyte subset, impairment of natural killer cell–dependent lysis of infected cells, impairment of humoral immunity, and effects of iron on “oxidative stress” and lipid peroxidation are possible mechanisms that could be involved in the iron overload status of the patients with hepatitis C.

The second group of patients in our study included individuals in whom the cause of cirrhosis could not be established by clinical and pathologic examination (cryptogenic cirrhosis). In contrast to the 11.5% of the patients with hepatitis C, only 4.5% of the patients with cryptogenic cirrhosis showed the HFE mutation. The patients with hepatitis C also showed a greater tendency for increased iron deposition in the liver. While 50% of the patients with cryptogenic cirrhosis were negative for iron staining, only 23% of the patients with hepatitis C were negative. The results of this study indicate that deposition of hepatic iron is a significant finding in the pathogenesis of hepatitis C cirrhosis, and the presence of heterozygous mutations of the HFE gene may further contribute to the increased iron deposition seen in these patients.

References

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