Environmental and genetic modifiers of the progression to fibrosis and cirrhosis in hemochromatosis

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Hereditary hemochromatosis is a genetic disorder of iron metabolism leading to inappropriate iron absorption and iron loading in various organs especially the liver. Despite the genetic mutation being relatively common in those of Anglo Celtic descent, cirrhosis of the liver occurs in only a small proportion of affected individuals. The risk of hepatic fibrosis and cirrhosis relates to the degree of iron loading with threshold hepatic iron concentrations being identified from population studies. However, other environmental and possibly genetic factors appear to modify this risk. Excess alcohol consumption appears to be one of the most important cofactors with steatosis and coexistent viral infection also implicated. Genetic polymorphisms in genes associated with fibrogenesis, antioxidant activity, and inflammation have been investigated in several different forms of chronic liver disease. The variability in the expression of these genes that predispose patients with hemochromatosis to increased risk of severe liver disease is the subject of ongoing investigations. Clearly the progression of iron loading to cirrhosis marks a crucial stage in the natural history of a patient’s disease and therefore therapy and prognosis. This review explores recent developments in knowledge of environmental and genetic modifiers of this process. (Blood. 2008;111:4456-4462)

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Introduction

Despite recent advances in the understanding of normal and abnormal iron metabolism, hemochromatosis remains an enigmatic disease. The original description more than 100 years ago by von Recklinghausen of advanced iron deposition within tissues and end organ damage is now an uncommon presentation, although the HFE genetic mutations are among the most common in Northern European races.1

The identification of the HFE gene2 simplified genetic testing for hemochromatosis. However, the subsequent recognition of the incomplete penetrance of the condition has led to a search for genetic and other modifiers of clinical expression. Liver disease is one of the hallmarks of hemochromatosis, and it is now clear only a proportion of those genetically predisposed C282Y homozygotes will ever have evidence of hepatic fibrosis.3 The point at which iron burden plus cofactors trigger the development of hepatic fibrosis and progression to cirrhosis and its complications is a critical stage in the patient’s prognosis.4 Knowledge of the processes and influences on the progression of liver fibrosis in many different diseases has progressed rapidly; therefore it is pertinent to examine these with respect to hemochromatosis.

Penetrance

The disease model for classic hemochromatosis involves a stepwise process from the genetic mutation in HFE (C282Y or H63D), to an elevated transferrin saturation, tissue deposition of iron associated with an increased serum ferritin concentration, and the potential for progression to hepatic fibrosis and cirrhosis and other organ damage. The penetrance is the proportion of individuals who develop manifestations of disease—which can be either biochemical or clinical both. The clinical penetrance of hemochromatosis is a subject of current controversy.

It has been demonstrated that most C282Y homozygotes will have evidence of expanded iron stores suggesting a high biochemical penetrance for the genetic abnormality. In population-based screening studies it has been shown that between 75% and 94% of all homozygotes will have an elevated transferrin saturation and 64% to 68% will have an increased serum ferritin level.3,5-8 In all studies, males have been shown to be more susceptible than females.

However, the risk of clinical disease within these populations is more uncertain, particularly as many of the symptoms such as fatigue, arthralgia, and impotence are common in the general population, therefore studies without a control group become difficult to interpret. Cirrhosis can occur in the absence of symptoms9 or elevated liver enzymes10 and those at risk due to heavy iron loading (serum ferritin > 1000 µg/L) or age (> 40 years) require liver biopsy to exclude hepatic fibrosis.11 Only a minority of population studies systematically biopsied C282Y homozygotes subjects at risk of cirrhosis and reported the results.

Olynuk et al had biopsy results available in 11 of 16 homozygous subjects and found that one was cirrhotic in the context of excessive alcohol use.7 Three had fibrosis evident suggesting that overall 25% may have been at significant risk of progressive liver disease. In a study of 65 238 Norwegian subjects assessed phenotypically, 269 were thought to have hemochromatosis on the basis of clinical and laboratory evidence.12 Twelve (11%) of 106 available liver biopsies had at least moderate fibrosis present. Allen et al demonstrated hepatic fibrosis or cirrhosis in 7% of their overall cohort of 158 C282Y homozygotes including at least 15%
of males. Beutler et al used plasma collagen IV concentrations as a marker for hepatic fibrosis and found that 25.8% of homozygotes exceeded the upper limit of normal compared with 11.1% from the control population. A referral and family-based study by Powell et al found stage II or greater fibrosis in 18% of the male patients under review and 5.6% were cirrhotic.

While up to 25% of patients newly diagnosed with iron-induced cirrhosis will have evidence of portal hypertension, this can regress with venesection therapy. The incidence of hepatocellular carcinoma (HCC) however remains elevated compared with patients with cirrhosis from other causes, although this could be affected by the duration of disease. HCC remains a significant cause of death with cirrhosis from other causes, although this could be affected by the duration of disease. HCC remains a significant cause of death

In summary, of those genetically predisposed individuals homozygous for the C282Y mutation, 75% to 100% of males will have biochemical evidence of increased iron stores and up to 10% to 25% of males may have fibrosis on biopsy, while 4% to 6% will have frank cirrhosis. The proportion of affected females has been shown to be much lower.

### Threshold hepatic iron concentration

To determine the threshold iron level that leads to an increased risk of hepatic fibrosis, several groups have studied hepatic iron concentration (HIC) in conjunction with histologic features from liver biopsy and produced receiver operating characteristic (ROC) curves. One such study used 100 C282Y homozygotes and excluded those with cofactors for liver injury. Using an analysis for the presence or absence of cirrhosis, a threshold HIC of 283 μmol/g dry weight (dw) was obtained. In another study, a similar threshold HIC of 236 μmol/g dw demonstrated optimum sensitivity (80%) and specificity (78%) for detecting cirrhosis. O'flynn et al postulated that the duration of iron overload may be important in determining hepatic fibrosis. It was found that the product of HIC and age performed better in diagnosing high-grade fibrosis (grades 3 and 4). A cutoff value of 479 745 μg/g × years (8566 μmol/g × years) provided a sensitivity of 100% and specificity of 86%.

Although these predictors of risk are useful, there is still considerable overlap such that not all subjects with threshold iron loading will have cirrhosis and others will be cirrhotic despite a HIC below these values. The risk of an individual developing hepatic fibrosis as a result of the HFE mutation therefore is likely to depend on the interplay between environmental factors and genetic influences. These may be divided into those that affect the degree of iron loading and those affecting the risk of progressive hepatic fibrosis.

### Factors affecting iron loading

#### Environmental

The underlying genetic defect in hereditary hemochromatosis leads to inappropriately increased transport of iron from the duodenum. It could be expected that C282Y homozygotes with environmental cofactors decreasing iron absorption or increasing loss may be protected from disease penetrance, however the available evidence suggests this effect is small (Table 1).

A link between alcoholic liver disease and iron overload has long been observed and experimental work has helped to reveal the relationship. Animal studies have demonstrated that alcohol consumption is associated with a down-regulation in hepcidin mRNA expression in liver tissue leading to increased expression of iron transport molecules DMT1 and ferroportin. This effect can be abolished in vitro by blocking the alcohol metabolizing enzymes. Antioxidants ameliorated the effect of alcohol on hepcidin and downstream proteins, thus suggesting oxidative stress as a mechanism contributing to iron loading.

#### Sex

There is therefore evidence to suggest that environmental factors play a minor role in determining the degree of iron loading. At present, however, population studies would suggest that the greatest influence on biochemical penetrance remains female sex particularly in the premenopausal state. It has been shown that women who have a menopause or hysterectomy before the age of 50 years will have a higher HIC than those undergoing this at an older age. The same effect has not been demonstrated for pregnancy, with the number of pregnancies showing no correlation with iron burden in C282Y homozygote women.

The protective effect of female sex has frequently been attributed solely to physiological blood loss, however there is some evidence to suggest genetic factors may be involved. Early genetic studies prior to cloning the HFE gene demonstrated that males homozygous for the D6S105 allele 8 on chromosome 6 had a propensity to heavier iron loading but this effect was not seen in females. Recent observations from Barton et al show a disparity between the HLA A and B haplotype frequencies in male and female hemochromatosis patients, which appears to also occur in other chromosomes 6p-linked disorders.

Further, the CD8+ T lymphocyte profile has also been identified as a modifer of iron stores but this effect is not seen in females. Given these findings, it is tempting to speculate on the presence of further modifying genes on chromosome 6 that have a sex-specific effect on iron loading and are associated with a locus controlling T-cell numbers. Of interest, recent reports suggest that this potential sex-linked
phenotype disparity may extend beyond HFE and chromosome 6 with a demonstrated increase in the frequency of the Q248H polymorphism in the ferroportin-1 gene in African-American males with elevated serum ferritin levels. Wang et al used a mouse model to identify a male predisposition for the expression of Mon1a (involved in trafficking cell surface and secreted molecules), which plays a role in ferroportin trafficking. Mutations in this gene result in altered macrophage iron levels.

**Genetic**

A growing appreciation of the phenotypic heterogeneity of HFE-hemochromatosis has prompted investigations into other genes that could modify iron loading. The molecular players in the field of iron homeostasis have increased in number as knowledge has advanced. It is now recognized that hepcidin is the central regulator, acting to down-regulate iron absorption via binding to ferroportin and causing its internalization and degradation within enterocytes and macrophages. As ferroportin is responsible for the transport of iron across the basolateral enterocyte membrane, this reduces the supply of iron to the extracellular space. The molecular regulation of hepcidin expression is more complex with HFE, transferrin receptor 2, hemojuvelin (HJV), and bone morphogenetic proteins (BMP) the key factors involved. Serum HJV levels increase in response to iron deficiency with an early rise due to shedding from the cell membrane. This release requires the membrane protein neogenin and is mediated through transferrin.

It has been recognized that iron overload can occur secondary to mutations in these iron regulatory proteins, thus it is an attractive proposal that the inheritance of a single mutation in these loci could give rise to a more severe phenotype in subjects with abnormalities in the HFE gene. This has been borne out in several studies (Table 2). Until recently, it appeared that the proportion of patients with other identifiable genetic variants was small. The recent discovery of more common polymorphisms in the BMP signaling pathway provides an alternative explanation for the variability in iron loading and has a clear physiological basis via the central molecule hepcidin.

While hepcidin is accepted as the central molecule controlling iron absorption, there is some evidence to suggest the existence of hepcidin-independent mechanisms involving the enterocyte. Exposure of the enterocyte to dietary iron produces a rapid reduction in mRNA encoding the apical iron transporting molecule DMT1 without affecting ferroportin on the basal cell surface (reviewed in Oates). This suggests the effect is not mediated through hepcidin but may relate to changes in the intracellular iron pool. At present, it is unclear whether this pathway plays any role in explaining the heterogeneity of iron loading in hemochromatosis.

While there are clearly a number of factors that influence the degree of iron loading seen in those genetically predisposed individuals homozygous for the C282Y mutation, it appears that not all liver disease in these patients is due to iron alone. Rather, there is likely to be a threshold of iron loading above which hepatic fibrosis is more likely to occur, and this threshold may well be affected by other environmental and genetic factors.

**Factors predicting progression to liver disease**

**Environmental**

*Alcohol.* The acceleration of liver injury in hemochromatosis patients consuming substantial amounts of alcohol is widely recognized. In one study of well-characterized hemochromatosis subjects, it was found that alcohol in excess of 60 g/day was associated with a much greater incidence of cirrhosis (61.1% vs 7%), and the mean HIC at which cirrhosis developed was lower in drinkers than nondrinkers. It was also noted that the age at which cirrhosis occurred tended to be lower in the group consuming excess alcohol. Adams and Agnew found a similar effect when they studied 16 hemochromatosis patients who consumed more than 80 g alcohol/day and compared these to HLA-identical siblings who were not drinking excessively. Again, cirrhosis was more common in drinkers (7/16 versus siblings 0/20), although it was apparent that the drinkers also had increased serum ferritin levels and exchangeable body iron and therefore heavier iron loading may have also contributed to the increased incidence of cirrhosis.

Taken together, these and other studies provide strong evidence for the role of alcohol as a cofactor in the progression to hepatic fibrosis and cirrhosis. There is a biochemical explanation for this given that both alcohol and iron produce hepatic oxidative stress and there is experimental evidence to suggest that these effects are cumulative.

*Hepatic steatosis.* There is established evidence that the metabolic syndrome (central obesity, insulin resistance, dyslipidemia) is a strong risk factor for hepatic steatosis and that steatosis may accelerate other chronic liver disease such as chronic hepatitis C. The role steatosis may play in predisposing to fibrosis progression in hemochromatosis has now been investigated in a review of 214 patients homozygous for the C282Y mutation who had all undergone liver biopsy. In this study, there was a significant correlation between steatosis on biopsy prior to venesection and the presence of fibrosis. This relationship remained significant after adjustment for other factors such as alcohol and iron loading (HIC). It has also been noted that venesection can improve insulin resistance in nonalcoholic fatty liver disease. Thus it appears a relationship exists between iron, steatosis, insulin, and liver fibrosis. While the mechanisms that cause accelerated liver disease progression remain to be elucidated, the additive effects of iron- and steatosis-induced oxidative stress and lipid peroxidation may offer a potential explanation.

*Viral hepatitis.* There have been a number of studies investigating whether HFE gene mutations may alter the course of chronic
hepatitis C, and methodologies have varied as have the conclusions. In a large cohort of patients with chronic hepatitis C and controlling for duration of infection, HFE mutations were found to be independently associated with bridging fibrosis or cirrhosis in those with compensated liver disease, suggesting a role in the acceleration of fibrosis progression.50 There is some evidence to suggest that elevated hepatic iron may lead to poorer responses to hepatitis C virus (HCV) treatment51 and that adjuvant venesection may improve sustained virologic response rates in patients treated with interferon and ribavirin.52

There have been relatively few studies assessing the reverse situation—that is, populations of hemochromatosis patients to determine whether the coexistence of viral hepatitis predisposes to more advanced hepatic disease. In one such retrospective review, patients with HCV and hemochromatosis were compared with those with either condition alone.53 All patients were selected to have advanced fibrosis or cirrhosis. It was concluded that the age at presentation with cirrhosis was significantly younger in those with the 2 conditions combined and the HIC at which this occurred was significantly lower, thus supporting the concept of viral hepatitis being synergistic in causing liver injury.

**Genetic**

**Sex.** Population studies have consistently shown that male sex is a risk factor for hepatic fibrosis in hemochromatosis3,7,9,12,54 and this has been presumed to relate to greater iron burden (Table 3). There is some limited evidence to suggest that men may have a greater risk even when liver iron concentration and alcohol intake are not different between sexes.65 It has been shown in a multiple regression analysis that the rate of fibrosis progression in hepatitis C is independently related to male sex,66 and in a retrospective study of women with chronic hepatitis C, estrogen exposure was protective against fibrosis progression.67 It is possible therefore that females with hemochromatosis are protected not only through physiological blood loss but also through an antifibrotic effect of estrogens, which have been shown to have an antioxidant effect in the liver.68 This effect would be expected to be lost after menopause.

**Proinflammatory cytokines.** Hepatic stellate cell activation is known to be a critical step in the initiation of hepatic fibrosis.66 Monocyte chemotactic protein 1 (MCP-1) is a chemokine involved in the recruitment of monocytes and T lymphocytes and is secreted by a variety of cells. It has been shown that activated hepatic stellate cells contribute to the expression and respond to the chemotactic signal of MCP-1.71,72 It is present in small amounts in normal liver tissue, but expression becomes markedly up-regulated in chronic hepatitis and cirrhosis.71 A polymorphism has been identified in the promoter region of the gene at position −2518 relative to transcription start, which affects MCP-1 gene expression.73 This polymorphism has not been studied in hemochromatosis subjects, however, one study in chronic hepatitis C patients has found the A/A genotype to be more common in those with lesser degrees of fibrosis and inflammation, indicating a potential protective effect. This genotype was shown to correlate with lower levels of MCP-1 mRNA in hepatic specimens and less MCP-1 secretion from cultured hepatic stellate cells after stimulation.55 Conflicting results have been found by other groups in both smaller63 and larger64 studies.

**Oxidative injury.** It is proposed that the cellular injury in iron-overloaded livers relates to the generation of hydroxyl radicals and subsequent peroxidation of lipid membranes and organelles.80
The hypothesis that genetic polymorphisms in antioxidant enzymes may have a phenotypic effect is attractive. Glutathione transferases catalyze conjugation reactions, inactivating toxic products and reactive oxygen species. A polymorphism in glutathione S transferase P1 (GSTP1) results in an amino acid substitution in codon 105 (Ile→Val) producing reduced functional activity. This less active allele has been shown to be associated with cryptogenic liver cirrhosis and the Val/Val genotype was significantly more frequent in cirrhotic individuals than noncirrhotics, and the association remained significant when sex, age, and ferritin levels were also considered in a logistic regression analysis.

Myeloperoxidase (MPO) is an enzyme present in neutrophils and monocytes and is responsible for catalyzing the reaction to produce hypochlorous acid to effect cell damage. It has been theorized that hepatocellular injury from cellular immune responses and the generation of reactive oxygen species could lead to cell stellate cell activation in liver disease. The recognition of a single nucleotide polymorphism in the promoter region of the MPO gene leading to variation in the binding site and mRNA levels led to this region being studied with respect to hemochromatosis expression. The results from this study did indeed show an association with the G/G genotype producing greater MPO activity being statistically more common in the cohort of cirrhotic C282Y homozygotes. This association remained significant on multivariate analysis.

Conclusions

Hemochromatosis is a disease related to a relatively common and easily identifiable genetic defect, but the extent of clinical expression is variable and to a large extent remains unexplained. Biochemical evidence of disordered iron metabolism is frequent especially in males, but far fewer develop the potentially severe end organ disease such as liver cirrhosis. Alcohol consumption would appear to remain the single most important environmental cofactor leading to liver disease, and the recent work describing the interaction between iron, alcohol, and the liver is exciting.

Analyses of genetic polymorphisms have become simpler to perform, however many such studies suffer from small sample sizes, thus risking misleading results. While polymorphisms of single nucleotides have been studied, perhaps the most important genetic influence is determined by the presence of the Y chromosome. Further information should emerge as the process of hepatic fibrogenesis is characterized and the polygenic variation between patients is better defined.

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