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Evidence That the Ancestral Haplotype in Australian Hemochromatosis Patients May Be Associated with a Common Mutation in the Gene

D. H. G. Crawford, L. W. Powell, B. A. Leggett, J. S. Francis, L. M. Fletcher, S. I. Webb, J. W. Halliday, and E. C. Jazwinska

Joint Liver Program, The Queensland Institute of Medical Research and Department of Medicine, The University of Queensland, Brisbane

Summary

Hemochromatosis (HC) is a common inherited disorder of iron metabolism for which neither the gene nor biochemical defect have yet been identified. The aim of this study was to look for clinical evidence that the predominant ancestral haplotype in Australian patients is associated with a common mutation in the gene. We compared indices of iron metabolism and storage in three groups of HC patients categorized according to the presence of the ancestral haplotype (i.e., patients with two copies, one copy, and no copies of the ancestral haplotype). We also examined iron indices in two groups of HC heterozygotes (those with the ancestral haplotype and those without) and in age-matched controls. These analyses indicate that (*i*) HC patients with two copies of the ancestral haplotype show significantly more severe expression of the disorder than those with one copy or those without, (*ii*) HC heterozygotes have partial clinical expression, which may be influenced by the presence of the ancestral haplotype in females but not in males, and (*iii*) the high population frequency of the HC gene may be the result of the selective advantage conferred by protecting heterozygotes against iron deficiency.

Introduction

Hemochromatosis (HC) is a common disorder of iron metabolism inherited as an autosomal recessive trait and characterized by an inappropriate increase in iron absorption and a progressive increase in body iron stores (Powell et al. 1990). In the absence of pathological or excess physiological blood loss, and with adequate iron intake, virtually all subjects homozygous for the HC defect will show phenotypic expression of the disease with progressive iron loading of the liver and other organs (Powell et al. 1990). However, there is a wide variation in the clinical features in HC, particularly with respect to the two measures of hepatic iron loading: hepatic iron concentration (HIC) and hepatic iron index (HII = HIC/age in years) (Muir et al. 1984). The variation in clinical expression cannot be fully accounted for by differing exposure to environmental factors (Crawford et al. 1993), thus genetic factors are likely to be important determinants of the clinical variation in HC.

Of the biochemical markers of hepatic iron loading, HII—rather than HIC—must be regarded as the most accurate measure of clinical expression of the underlying genetic defect, because the absolute hepatic iron concentration is age dependent (Powell et al. 1990). The HC gene was first shown to be linked to the HLA class I genes on chromosome 6 by Simon et al. (1976), and this location enables class I typing to be used for gene tracking in affected pedigrees. More recently we have shown that the HC gene is likely to lie in a 3,000-kb region of 6p that includes the following five highly polymorphic markers: D6S248, D6S265, HLA-A, HLA-F, and D6S105. All of these markers show significant allelic association with HC, and a specific combination of the alleles at these markers defines the common predominant ancestral haplotype in Australian patients (Jazwinska et al. 1993, 1995). This ancestral haplotype is found on 33% of affected chromosomes, and it is uniquely associated with HC: no other haplotype is seen to cluster in Australian patients (Jazwinska et al. 1995). In other inherited diseases, haplotypes have been found to be in linkage disequilibrium with different mutations at the disease-gene locus, and specific mutations have been found to account for the variable phenotypic expression of the underlying disease (Kerem et al. 1990; Petrukin et al. 1993; Sheppard et al. 1993; Thomas et al. 1995). Thus, the ancestral haplotype in the Australian HC population may be associated with a common mutation of the HC gene that may define clinical expression.

Clinical expression of HC in heterozygotes is controversial, for, while it has been shown that HC heterozygotes do not develop the symptom-complex associated with HC (Powell et al. 1990), some studies have

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Address for correspondence and reprints: Dr. Elizabeth C. Jazwinska, The Liver Unit, The Queensland Institute of Medical Research, P. O. Royal Brisbane Hospital, Brisbane 4029, Australia. E-mail: lizJ@qimr.edu.au
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reported that ~30% of HC heterozygotes show partial biochemical expression, as evidenced by an increase in serum ferritin concentration (SF) or transferrin saturation (TS) (Cartwright et al. 1979; Summers et al. 1990; Adams 1994). The number of patients in each study has generally been small, and results have been variable. Furthermore, in virtually all of the previous studies reporting iron indices in HC heterozygotes, no adjustments have been made for age and gender, factors that influence SF (Leggett et al. 1990). Nevertheless, it remains possible that some HC heterozygotes do exhibit partial phenotypic expression of the disease, and it is also possible that partial expression is associated with a specific mutation at the HC gene locus.

The aims of this study were to address the issue of clinical expression of HC and its relation to the ancestral haplotype. Indices of iron metabolism were examined with respect to the presence of the ancestral haplotype in HC patients and HC heterozygotes. In the HC heterozygotes, measurements of the SF and TS were also compared with those of a large cohort of age-and gender-matched controls, to further examine whether there is partial biochemical expression in heterozygotes.

Material and Methods

HC Patients

Detailed haplotype analysis and quantitative assessment of body iron stores were performed in 129 HC patients. The diagnosis of HC was established in each patient by the presence of at least two of the following criteria, in the absence of known causes of secondary iron overload: (*a*) HIC >80 μmol/g dry weight; (*b*) HII >2; (*c*) stainable iron in the liver (grade 3–4); and (*d*) >4 g of iron removed by quantitative phlebotomy.

HC Heterozygotes

Gene tracking by HLA-A and -B typing in affected pedigrees identified 171 putative HC heterozygotes. A further 85 subjects were regarded as obligate heterozygotes being asymptomatic parents of a known HC patient.

Controls

TS and SF concentration of 582 employees (305 males and 277 females) of a large Australian banking corporation were measured. Data from this group of healthy volunteers formed part of a previously published study of biochemical indices of iron metabolism in the Australian population (Leggett et al. 1990).

Haplotype Marker Analysis

Five highly polymorphic markers, D6S248, D6S265, HLA-A, HLA-F and D6S105, in the HC gene region were analyzed in HC patients. HLA-A and B typing was performed by standard lymphocytotoxicity methods (Doran et al. 1981). Microsatellites (D6S248, D6S265, D6S105, and HLA-F) were amplified from 100 ng of genomic DNA as described elsewhere (Jazwinska et al. 1993, 1995).

The full ancestral haplotype is characterized by the alleles 5-1-3-2-8 at the five highly polymorphic markers (marker order D6S248, D6S265, HLA-A, HLA-F, D6S105). Our previous analyses clearly showed that all patients who have HLA-A3 not associated with HLA-B35 have the full ancestral haplotype (Jazwinska et al. 1995). In the current study, patients who were homozygous for HLA-A3 were typed for all microsatellite markers. This confirmed their homozygous ancestral haplotype status and further confirmed the finding that in Australian HC patients the only HLA-A3 that is not associated with the ancestral haplotype is HLA-A3 on a haplotype with HLA-B35. In all other patients, microsatellite haplotype arrangements could not be defined directly, without extensive pedigree analyses, thus HLA-A3 in association with HLA-B alleles 18, 27, 40, 44, 51, 55, 57, and 62 was then used as the marker for ancestral haplotype status in all other patients. Patients were classified into three groups according to their ancestral haplotype status: those with two copies (as defined by microsatellite typing), those with one copy (as defined by the presence of HLA-A3 not in association with HLA-B35), and those with no copies (who carried HLA-A3 with HLA-B35 or who were HLA-A3 negative). The HC heterozygotes were classified into two groups: those who carried the ancestral haplotype (as defined by HLA-A3 without HLA-B35 on one chromosome) and those without the ancestral haplotype (who were HLA-A3 with HLA-B35 or HLA-A3 negative).

Assessment of Iron Status and Liver Histology

TS and SF were measured in all subjects by standard techniques. HIC was measured by atomic absorption spectrophotometry following acid digestion, as described elsewhere (Bassett et al. 1986). HII was calculated by dividing HIC (μmol/g dry weight) by the patient's age in years (Summers et al. 1990). Liver biopsy sections were stained with hematoxylin, eosin, and Perls's Prussian blue stain, using standard techniques.

Statistical Analysis

Measurements of TS, HIC, and HII were expressed as mean ± SD and were compared between groups of subjects by analysis of variance (ANOVA). Student's *t* test with Bonferroni's correction was applied to the data, when significant differences were detected by ANOVA. The distribution of SF values was skewed to the right, the median and range of SF was thus reported, and the logarithmic transformation was applied and Student's *t* test was used to compare the mean values of groups.

Table 1

Haplotype Arrangements in the 14 HC Patients Homozygous for the Ancestral Haplotype

Haplotype for D6S248–D6S265–HLA-A–HLA-F–D6S105	No. of Chromosomes
5–1–3–2–8	18
6–1–3–2–8	2
7–1–3–2–8	2
4–1–3–2–8	3
19–1–3–2–8	2
5–1–3–2–7	1

Because of the effect of physiological blood loss on body iron stores in females, a further analysis was performed in male patients only. Serum measures of iron metabolism are known to vary according to both age and sex, so analyses of these measures in controls and heterozygotes were carried out in males and females according to age. Comparison of the frequency of iron deficiency in patients and controls was made by χ^2 analysis.

Results

Presence of the Ancestral Haplotype in HC Patients

Because of the mechanism of slippage, microsatellites can have a high mutation rate; thus, those individuals in which a haplotype differed from the ancestral haplotype by one repeat at one microsatellite marker were considered to be carrying the ancestral haplotype (table 1). Of the 129 HC patients studied (82 males and 47 females), 10 (6 males and 4 females) were homozygous for the ancestral haplotype. Two males and two females carried a haplotype in which D6S248 differed by more than one repeat from the ancestral allele. As D6S248 is the most proximal marker of the haplotype (Jazwinska et al. 1995) and is situated between HLA-A and HLA-B, where recombinations have been reported in HC (Jazwinska et al. 1993), these four haplotypes were also considered to be of ancestral origin. All haplotypes classified as ancestral are given in table 1. Thirty-seven patients (21 males and 16 females) carried the ancestral haplotype on one chromosome and 78 patients (53 male and 25 female) did not carry the ancestral haplotype on either affected chromosome.

Iron Indices in HC Patients

The mean HII was found to be increased in patients homozygous for the ancestral haplotype compared with those who had only one copy of the ancestral haplotype ($P < .003$) and those who did not carry the ancestral haplotype ($P < .003$) (table 2). However, there was no significant difference in mean TS, SF, or mean HIC among the three groups of patients.

Iron Indices in Male HC Patients

The HII was higher in male patients who were homozygous for the ancestral haplotype than in male patients who carried one copy of the ancestral haplotype ($P < .003$) and male patients who did not carry the ancestral haplotype ($P < .003$). The measurements of HIC, TS, and SF were, however, similar in all the three groups of male patients (see table 2).

Iron Indices in HC Heterozygotes

The HC heterozygote group consisted of 135 males and 121 females. The mean TS was significantly greater in the HC heterozygotes than in the control population (34.5% ± 14.3% vs 25.2% ± 12.2%; $P < .001$) (table 3). This difference was present in both females (33.1% ± 12.1% vs 23.8% ± 10.2%; $P < .001$) and males (35.8% ± 13.5% vs 26.1% ± 15.5%; $P < .001$). In contrast, there was no difference in SF concentration between HC heterozygotes and controls in any decade of life. As expected, in HC heterozygotes and controls, the SF concentration was higher in males than in females in every decade of life except in the <20-year age group. The relationship between increasing age and SF concentration was similar in HC heterozygotes and in controls. In control males and HC heterozygote males, SF increased with age until the 5th decade, after which it reached a plateau. In HC heterozygote females and control females, SF increased after the age of 50 years—consistent with the reduction in physiological blood loss after the menopause.

When female HC heterozygotes were considered, the SF was significantly increased in those with the ancestral haplotype, compared with those without the haplotype ($P < .012$; median values 112 μg/liter vs 67 μg/liter), while TS was similar in the two groups (32.6% ± 16.5% vs 30.7% ± 12.7%). Only 1 of the 121 heterozygote females was iron deficient (SF <10 μg/liter), compared with 14 of the 263 female controls ($\chi^2 = 4.20$; $P < .05$).

When only the male HC heterozygotes were considered, the presence of the ancestral haplotype did not appear to influence iron indices. The mean TS and the median SF in those with and without the haplotype were 35.3% ± 11.5% versus 34.9% ± 12.9% and 249 μg/liter versus 246 μg/liter, respectively. One of the 135 male HC heterozygotes and 2 of the 305 male controls were iron deficient (i.e., SF < 10 μg/liter).

Discussion

This study addresses the relationship between the ancestral haplotype and clinical expression of HC. The results provide evidence that Australian HC patients, homozygous for the ancestral haplotype, accumulate hepatic iron at a faster rate (as measured by HII), and thus express a more severe form of the disorder than other

Table 2

Iron Indices in Hemochromatosis Patients according to Ancestral Haplotype (AH) Status

	No.	SF (μg/liter)	TS (%)	HIC (μmol/g)	HII (HIC/age in years)
All patients:					
No AH	78	973	80.0 ± 16.6	228 ± 116	5.4 ± 3.2
One AH	37	700	79.7 ± 17.6	189 ± 160	5.0 ± 2.9
Two AH	14	1,030	88.4 ± 7.8	302 ± 149	9.1 ± 4.5[a]
Male patients:					
No AH	53	1,000	78.9 ± 15.6	240 ± 157	5.6 ± 3.3
One AH	21	905	80.8 ± 17.5	188 ± 170	5.0 ± 3.1
Two AH	8	1,030	89.4 ± 6.2	318 ± 176	10.1 ± 4.6[a]

NOTE.—SF = serum ferritin (median); TS = transferrin saturation (mean ± SD); HIC = hepatic iron concentration (mean ± SD); HII = hepatic iron index (mean ± SD).

[a] $P < .003$ when compared with the other two groups.

patients. Because iron accumulation is age related (Summers et al. 1990), the HII rather than HIC must be regarded as the better marker of phenotypic expression. Indeed, the mean HII was ~50% greater in patients who were homozygous for the ancestral haplotype than in patients in the other two groups. The results of the analysis performed using male patients only clearly showed that male patients who are homozygous for the ancestral haplotype accumulate hepatic iron at a significantly greater rate than male patients who do not have two copies of this haplotype.

This study supports our previous analyses, in which we showed a concordance of iron storage between sibs with HC (Crawford et al. 1993). This previous study concluded that genetic factors are an important determinant of the rate of iron accumulation in HC, because the variation in HII could not be accounted for by environmental factors (Crawford et al. 1993). We also hypothesized that more than one mutation exists at the HC gene locus and that differing mutations account for the variable phenotypic expression of the disease. In other inherited diseases, microsatellite haplotypes have been found to be in linkage disequilibrium with different mutations at the gene locus (Petrukin et al. 1993). Thus, the ancestral haplotype in the Australian HC population is likely to represent a common mutation at the HC gene locus, and this mutation appears to be associated with more severe clinical expression, as evidenced by the significantly greater rate of iron accumulation.

Table 3

Median Serum Ferritin Concentration and Range of Values in Heterozygotes and Volunteers

Sex and Age Group (years)	Heterozygotes Median (range) [no.]	Volunteers Median (range) [no.]
Male:		
<20	65.5 (9–752) [32]	92 (20–465) [33]
20–29	165 (20–450) [25]	165 (6–1,011) [97]
30–39	200 (14–725) [26]	225 (39–793) [69]
40–49	306 (13–810) [21]	223 (15–1,550) [67]
50–65	294 (12–810) [31]	200 (34–470) [39]
Female:		
<20	45 (10–227) [17]	34 (3–137) [57]
20–29	36.5 (14–410) [28]	39 (2–333) [135]
30–39	82.5 (5–360) [19]	66 (5–312) [36]
40–49	62 (19–450) [25]	50 (5–514) [38]
50–65	117.5 (38–482) [32]	128 (37–356) [11]

This present study also examined iron indices in HC heterozygotes. TS was significantly increased in male and female HC heterozygotes when compared with controls. The values of TS reported here are similar to those described in a recent report of 255 heterozygote patients (Adams 1994). TS is a marker of iron delivery to tissues. In healthy individuals, TS reaches a peak during the 2d decade and is stable in the following years (Yip et al. 1984). Thus, there is no need to correct measurements of TS for the effects of increasing age. TS rises earlier in the course of iron overload than does SF or urinary-iron excretion following desferrioxamine administration (Edwards et al. 1977). It is thus of interest that the demonstration of an elevated TS in HC heterozygotes probably reflects altered ferrokinetics and partial phenotypic expression of the underlying biochemical abnormality. In contrast, both age and gender have a significant influence on values of SF. In healthy males, SF increases progressively from 20 to 50 years of age. Healthy females have a significantly lower SF than males but show a rise in the 5th decade—consistent with the cessation of menses. Thus, it is important that gender and age are considered when measurements of SF are compared between HC heterozygotes and healthy controls. Of particular interest was the lack of any difference in SF between the HC hetero-

zygotes and the control population, when age and gender corrections were made. In one other study of HC heterozygotes, appropriate adjustments for gender and increasing age were made (Cartwright et al. 1979), and this also showed that the values for SF concentration did not differ from those in controls. Thus, although the elevated TS suggests some abnormality of iron metabolism in HC heterozygotes, the normal SF indicates the mechanisms that limit iron accumulation in healthy subjects are still functional when one normal copy of the HC gene is present. It was also of interest that, when SF was analyzed in male and female HC heterozygotes according to the presence of the ancestral haplotype, a significantly higher SF was found in female HC heterozygotes who had the ancestral haplotype, but no significant difference was noted in males.

The ideal control subjects for these analyses are those defined as homozygous unaffected by gene tracking, rather than controls from a general population, because ⩾10% of the general population in Australia is carriers for HC. However, the number of true homozygous normal subjects in our HC families is too small to provide an adequate age distribution to allow valid age-and gender-related comparisons. The use of such controls, however, would be expected to enhance rather than negate the findings reported here.

The results of this study also support the hypothesis that the presence of the HC defect may protect heterozygotes against iron deficiency. We found the prevalence of iron deficiency to be significantly less in female HC heterozygotes than in a female control population. It is possible that the high frequency of HC in the population is maintained by a selective advantage conferred by its role in protecting against iron deficiency and is not selected out of the genetic pool, because it is rarely fatal until the 6th decade of life. It was also of interest that female HC heterozygotes with the ancestral haplotype had a significantly higher SF than those without the haplotype.

In conclusion, this study lends strong support to the hypothesis that the ancestral haplotype in Australian patients is associated with a common mutation in the HC gene, which is an important determinant of the severity of clinical expression, as evidenced by the rate of hepatic iron accumulation. Furthermore, we have confirmed that HC heterozygotes do exhibit partial phenotypic expression of HC that is characterized by an increase in TS but not an increase in SF. Finally, heterozygosity for the HC gene may, in part, protect women from iron deficiency, and the high prevalence of this disease and the ancestral haplotype in Caucasians may be related to such a selective advantage.

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