ORIGINAL RESEARCH

Iron overload and cirrhosis in referred *HFE* p.C282Y homozygotes with normal transferrin saturation and elevated serum ferritin

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ABSTRACT

BACKGROUND: Elevated transferrin saturation (TS) is an imperfect test to identify adults with high-iron gene (*HFE*) p.C282Y homozygosity or elevated hepatic iron concentration. **METHODS:** We analyzed observations of non-screening, previously untreated p.C282Y homozygotes who presented with both normal TS (<50% men, <45% women) and elevated serum ferritin (SF; men, >300 µg/L; women, >200 µg/L). Iron overload was defined as hepatocyte iron grade 3 or 4, liver iron >35 µmol/g dry weight, or iron removed by phlebotomy ≥3 g. Cirrhosis was defined as regenerating nodules of hepatocytes surrounded by bands of fibrous connective tissue. **RESULTS:** Among 917 referred p.C282Y homozygotes, 58 (33 men, 25 women) had normal TS and elevated SF (6.3% [95% CI 4.9% to 8.1%]). Of 58 patients, 14 (24.1%) underwent liver biopsy; all 14 had hepatocyte iron grade 3 or 4. Fatty infiltration was reported in 6 of 14 liver biopsies (42.9%). Liver iron was >35 µmol/g dry weight in 7 of 8 patients tested (87.5%). Iron removed by phlebotomy was ≥3 g in 75.0% (15/20) of men and 62.5% (5/8) of women. Of 58 patients, 3 (5.2%) had iron overload and cirrhosis; each also had a proven or possible non-iron liver condition that may have acted in synergy with liver iron to increase cirrhosis risk. **CONCLUSIONS:** Iron overload is common in non-screening, previously untreated *HFE* p.C282Y homozygotes with normal TS and elevated SF. Among our sample, 5.2% had cirrhosis. Clinicians should not assume that patients with normal TS and elevated SF do not have *HFE* p.C282Y homozygosity, iron overload, or cirrhosis.

KEYWORDS: cirrhosis; hemochromatosis; *HFE* p.C282Y homozygosity; transferrin saturation

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INTRODUCTION

The diagnosis of hemochromatosis has evolved since discovery of the high-iron gene (*HFE*) and development of a simple blood test for *HFE* p.C282Y (rs1800562). Before *HFE* mutation analysis was available, hemochromatosis was suspected in patients with elevated serum transferrin saturation (TS) and serum ferritin (SF). After *HFE* mutation analysis became available, the utility of

TS and SF testing was reported by referral centres and in pedigree studies and clinical guidelines (1,2). Our informal observations suggest that many clinicians associate elevated TS with *HFE* p.C282Y homozygosity and hemochromatosis. Nonetheless, the sensitivity of TS for diagnosis of p.C282Y homozygosity was 82% in previously untreated men (TS ≥50%) and 73% in previously untreated women (TS ≥45%) in the population-based Hemochromatosis and Iron Overload Screening (HEIRS) Study of 101,168 participants (3). Among white HEIRS Study participants with p.C282Y homozygosity, only 83% of previously untreated men and 75% of previously untreated women had elevated TS (4). Some previously untreated HEIRS Study participants with *HFE* p.C282Y homozygosity had normal SF and were considered to be non-expressing cases. Other participants with p.C282Y homozygosity had normal or elevated TS and elevated SF, although further investigation and follow-up was beyond the scope of the HEIRS Study (3). These observations suggest that some previously untreated p.C282Y homozygotes with normal TS levels have elevated SF levels and iron overload.

To learn more, we retrospectively studied the clinical profiles of non-screening, previously untreated *HFE* p.C282Y homozygotes with normal TS and elevated SF from two large tertiary hemochromatosis referral centres. We discuss our findings in the context of other pertinent associations of TS with iron overload, non-iron liver conditions, and cirrhosis in p.C282Y homozygotes.

METHODS

Patient selection

This work was performed according to the principles of the Declaration of Helsinki (5). Western University Health Science Research Ethics Board granted approval for performance of this study (HSREB file no. 102184 [PCA]). Western Institutional Review Board granted an exemption determination for performance of this study under 45 CFR 46.101(b)(4) (submission no. 2539985-44189619 [JCB]). Informed consent was not required because this study involved retrospective chart review and analyses of observations recorded during routine medical care.

We included all white patients referred to Ontario or Alabama tertiary centres for evaluation of hemochromatosis who were *HFE* p.C282Y homozygotes and who did not have iron deficiency (both TS <10% and SF <15 µg/L). *HFE* mutation analysis was performed for some patients by referring physicians and in all other referred patients by the authors to aid differential diagnosis of hemochromatosis and other liver disorders and meet expectations of referring physicians. Most referred patients were probands, although referred family members were included at the Ontario centre. All patients were previously untreated with phlebotomy or iron chelation therapy. Liver biopsy specimens were obtained and evaluated in referred patients before therapy, as clinically indicated. Patients were screened for chronic hepatitis B and C and were negative. We excluded previous HEIRS Study participants diagnosed as having p.C282Y homozygosity through screening (4,6).

Patient evaluation

We analyzed observations on *HFE* p.C282Y homozygotes who presented with both normal TS (men, <50%; women, <45%) and elevated SF (men, >300 µg/L; women, >200 µg/L). Using local clinical laboratories, TS (as a quotient of serum iron and total iron-binding capacity) and SF were measured in blood specimens obtained without regard to fasting, because fasting does not increase the sensitivity or specificity of TS for the detection of p.C282Y homozygosity (7). Repeat measurement of TS was not routinely performed as a pre-condition of performing *HFE* mutation analysis or after diagnosis of p.C282Y homozygosity. We defined Prussian blue-stained hepatocyte iron grade 3 or 4 as increased (8). The reference range for liver iron concentration measured by atomic absorption spectrometry was 0–35 µmol/g dry weight. Iron removed by phlebotomy to achieve iron depletion was estimated by the number of 500 mL phlebotomies × 0.25 g Fe/phlebotomy. Iron overload was defined as grade 3 or grade 4 hepatocyte iron (8), liver iron >35 µmol/g dry weight, or iron removed by phlebotomy ≥3 g (9). Cirrhosis was defined as regenerating nodules of hepatocytes surrounded by bands of fibrous connective tissue (10).

RESULTS

Among 917 referred p.C282Y homozygotes, 58 (6.3% [95% CI 4.9% to 8.1%]); 33 men, 25 women) had normal TS and elevated SF (Table 1). The proportions of men and women with normal TS and elevated SF were 6.3% and 6.4%, respectively.

Table 1: Normal TS and elevated SF in 58 referred *HFE* p.C282Y homozygotes with normal TS and elevated SF

Gender	Normal TS and elevated SF, *n* (%)*	SF, µg/L, *M* (range)	TS, %, *M* (range)	Age at diagnosis, y, *M* (range)	Iron removed, g, *M* (range), *n*	Patients with iron removed ≥3 g,† *n* (%)	Hepatic iron, µmol/g,‡ *M* (range), *n*	Cirrhosis, *n*
Men	33 (6.3)	1,079 (439–5,844)	39 (31–49)	50 (19–74)	7.1 (1.5–26), 20	15/20 (75)	257 (89–663), 6	2
Women	25 (6.4)	372 (202–793)	36 (13–43)	52 (29–77)	2.5 (0.5–3.5), 8	5/8 (63)	52 (29, 76), 2	1

**Normal TS: <50% for men, <45% for women. Elevated ferritin: >300 µg/L for men, >200 µg/L for women. These 58 patients represented 6.3% of 917 referred HFE p.C282Y homozygotes (527 men, 390 women).*
† *Iron overload was defined as ≥3 g iron removed by phlebotomy to achieve iron depletion.*
‡ *Reference range for liver iron is 0–35 µmol/g dry weight. Liver iron was >35 µmol/g dry weight in 7 of 8 patients so tested (87.5%).*
TS = transferrin saturation; SF = serum ferritin; HFE = high-iron gene

Of these 58 patients, 14 underwent liver biopsy (24.1%). All 14 patients who underwent liver biopsy had hepatocyte iron grade 3 or 4. Fatty infiltration was reported in 6 of 14 liver biopsies (42.9%). Liver iron was >35 µmol/g dry weight in 7 of 8 patients tested (87.5%). Iron removed by phlebotomy was ≥3 g in 75.0% (15/20) of men and 62.5% (5/8) of women (Table 1).

Of the 58 patients with normal TS and elevated SF, 3 (5.2%) had cirrhosis. The first, a man aged 56 years with SF 954 µg/L, had a history of excessive alcohol consumption; 10.5 g Fe was removed by phlebotomy before liver transplantation. The second, a man aged 75 years with SF 817 µg/L, had taken oral methotrexate weekly for arthritis. The third, a woman aged 56 years with SF 384 µg/L, had stainable hepatocyte iron grade 3, fatty liver, and sarcoidosis and hepatic granulomas; 1.6 g Fe was removed by phlebotomy (11).

DISCUSSION

We observed normal TS and elevated SF at presentation in 58 (6.3%) of 917 referred, previously untreated *HFE* p.C282Y homozygotes. The proportion of patients with normal TS and elevated SF was 6% in both men and women. In previously untreated white participants with p.C282Y homozygosity in the HEIRS Study, 6% of men and 26% of women had normal TS and elevated SF (4). Clinical guidelines promulgated by the European Association for the Study of the Liver (1) state that elevated TS and increased body iron stores are essential diagnostic criteria for *HFE* hemochromatosis. An algorithm regarding hemochromatosis diagnosis endorsed by the American Association for the Study of Liver Diseases does not include recommendations for the evaluation of persons with TS <45% and elevated SF (2). Our results demonstrate that a small but significant subgroup of non-screening, previously untreated p.C282Y homozygotes have normal TS and elevated SF. Their hemochromatosis diagnoses may have been overlooked with strict adherence to clinical guidelines.

Our results also demonstrate that iron overload defined by hepatic iron concentration or quantitative phlebotomy standards was present at diagnosis in 75% of previously untreated men and 63% of previously untreated women with p.C282Y homozygosity, normal TS, and elevated SF. In a study of 120 referred *HFE* p.C282Y homozygotes, TS >54% had sensitivity of 90% and specificity of 67% for elevated hepatic iron concentration. Pearson's correlation coefficient for TS versus hepatic iron concentration was only 0.29 ($P < .0001$) (12). SF in p.C282Y homozygotes may be elevated for reasons other than iron overload, including obesity, fatty liver disease, dysmetabolic iron overload syndrome, chronic alcohol use, chronic viral hepatitis, acute or chronic inflammation (including sarcoidosis), other liver conditions, and malignancy (11,13,14). Thus, these results confirm and extend previous reports that TS is a good but imperfect screening or diagnostic test for p.C282Y homozygosity (7) and hepatic iron concentration in p.C282Y homozygotes (12).

SF >1,000 µg/L is associated with increased cirrhosis risk in *HFE* p.C282Y homozygotes, although cirrhosis also occurs in p.C282Y homozygotes with SF ≤1,000 µg/L (15). Of 58 patients in this study, 3 (5.2%) with normal TS and elevated SF had cirrhosis, although their respective SF levels were

not especially high. Each of these 3 patients had a proven or possible non-iron liver condition that may have acted in synergy with liver iron deposition to enhance hepatic fibrogenesis and increase cirrhosis risk. In this study, 1 man with cirrhosis had a history of excessive alcohol consumption, a major risk factor for cirrhosis in *HFE* p.C282Y homozygotes (14). Another man with cirrhosis had taken methotrexate for arthritis management. In another study, a 58-year-old man took methotrexate therapy for psoriasis. Although he had elevated serum concentrations of hepatic enzymes, hepatic steatosis, and grade three hepatocyte iron, he did not develop cirrhosis (16). One woman in a previous publication had hepatic sarcoidosis (11). In this same publication, 5 of 7 patients with hemochromatosis and hepatic sarcoidosis also had cirrhosis (11).

TS is a composite of serum iron and transferrin levels. TS is significantly and independently associated with *HFE* p.C282Y (17–19). Elevated serum iron (and elevated TS) in p.C282Y homozygotes is typically attributed to increased export of iron from storage cells to plasma transferrin via ferroportin because of decreased hepcidin levels (20). It is assumed that iron absorbed from food also contributes to serum iron and TS in p.C282Y homozygotes (21). In 75 screening p.C282Y homozygotes with high TS–SF phenotypes, 4.0% had ferroportin gene (*SLC40A1*) Q248H, hepcidin gene (*HAMP*) –72C>T, or *HAMP* R59G heterozygosity, whereas none of these alleles was detected in 75 screening p.C282Y homozygotes with low TS–SF phenotypes (22). It has been proposed that other undefined mechanisms independent of hepcidin or p.C282Y also influence serum iron and TS in p.C282Y homozygotes (19). Taken together, these observations indicate that multiple factors promote elevated TS in p.C282Y homozygotes.

TF SNP rs3811647 (intron 11) was associated with significantly lower serum transferrin levels in adults unselected for hemochromatosis (23). Thus, it is plausible but unproven that rs3811647 negatively influences serum iron levels or TS in *HFE* p.C282Y homozygotes. In another report, five transferrin variants had no significant effects on TS, serum unbound iron-binding capacity, or SF in 113 persons with clinical hemochromatosis, and the prevalence of these variants in patients with hemochromatosis did not differ from that in the general population (24). Transmembrane protease, serine 6 (*TMPRSS6*) allele p.A736V (rs855791), has an estimated allele frequency in European whites of 0.34 (25) and was associated with lower serum iron levels and TS in adults unselected for hemochromatosis (23). Although we found no reports of rs855791 in p.C282Y homozygotes, $Hfe^{-/-}$ mice with heterozygous loss of Tmprss6 ($Hfe^{-/-}Tmprss6^{+/-}$) had significantly lower levels of serum iron, TS, and liver iron than $Hfe^{-/-}Tmprss6^{+/+}$ mice (26). In another study, analyses of genes that encode transferrin receptor-1, ferroportin, ceruloplasmin, ferritin light and heavy chains, iron regulatory proteins 1 and 2, and hepcidin in p.C282Y homozygotes revealed coding region and promoter polymorphisms, but none explained differences in TS or SF (27).

A strength of this study is the availability of a large cohort of non-screening, previously untreated *HFE* p.C282Y homozygotes from whom an accurate estimate of the proportion of patients presenting with normal TS and elevated SF could be made. Iron overload measurements of each type defined herein (stainable hepatocyte iron grade, liver iron concentration, and phlebotomy iron) were not available for all referred patients with normal TS and elevated SF. Thus, our prevalence estimate of iron overload in the 58 referred p.C282Y homozygotes is conservative. A limitation of this study is the lack of analyses that could permit association of acute or chronic inflammatory, autoimmune, or neoplastic disorders, transferrin variants, and *TF, TMPRSS6,* or other non-*HFE* alleles with TS and iron overload at presentation in non-screening, previously untreated *HFE* p.C282Y homozygotes.

Iron overload was common in non-screening, previously untreated *HFE* p.C282Y homozygotes with normal TS and elevated SF. Five percent had cirrhosis. Clinicians should not assume that patients with normal TS and elevated SF do not have *HFE* p.C282Y homozygosity, iron overload, or cirrhosis. *HFE* mutation analysis, a simple, inexpensive, and widely available blood test to detect p.C282Y, is a powerful diagnostic tool that improves evaluation of all patients with elevated SF suspected to have hemochromatosis, regardless of TS.

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