Research article

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

Effect of the A736V *TMPRSS6* polymorphism on the penetrance and clinical expression of hereditary hemochromatosis

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Background & Aims: Hereditary hemochromatosis (HH) is most frequently related to homozygosity for the p.C282Y *HFE* mutation ($C282Y^{+/+}$), hampering hepcidin induction in response to iron. The rs855791 polymorphism, encoding for the p.A736V variant of *TMPRSS6* regulating hepcidin, influences iron status in the population. The aim of this study was to assess the influence of rs855791 on the penetrance and clinical expression of HH.

Methods: We retrospectively considered 315 HH patients (163 $C282Y^{+/+}$, and 152 with other *HFE* genotypes) evaluated at the time of diagnosis, and 271 healthy controls with normal iron parameters, residents of Northern Italy; *TMPRSS6* genotype was assessed by allele specific polymerase chain reaction.

Results: The p.736V variant determining higher hepcidin release was under-represented in the patients ($p = 0.0023$), independently of the presence of the $C282Y^{+/+}$ genotype, and the p.736V/V genotype protected from HH independently of age and sex (OR of HH for p.736A/A: 2.57, 1.3–4.1 and for p.736A/V: 1.84, 1.1–3.2). In the 96 $C282Y^{+/+}$ male patients without chronic viral hepatitis and alcohol abuse, the "high hepcidin" p.736V allele was negatively associated with cirrhosis independently of age, ferritin, ALT levels, and alcohol intake (OR 3.93, 95% C.I. 1.17–14.61 for the p.736A variant), and with the cumulative incidence of hepatocellular carcinoma (17% p.736A/A, 4% p.736A/V, 0 p.736V/V, $p = 0.05$).

Conclusions: The p.A736V *TMPRSS6* polymorphism is likely a modifier of HH expression. Additional studies are warranted to validate these findings in other cohorts and test their potential relevance for the clinical management of HH patients.

Keywords: Cirrhosis; Genetics; Hepatocellular carcinoma; Hepcidin; Hereditary hemochromatosis; HFE; Iron; Penetrance; TMPRSS6.

Received 27 March 2012; received in revised form 4 July 2012; accepted 15 July 2012; available online 8 August 2012

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Abbreviations: HH, hereditary hemochromatosis; *HFE*, hemochromatosis gene; *$C282Y^{+/+}$*, homozygosity for the C282Y *HFE* mutation; HCC, hepatocellular carcinoma; TS, transferrin saturation; TMPRSS6, trans-membrane protease serine 6; IRIDA, iron-refractory iron-deficient anemia.

Introduction

Hereditary hemochromatosis (HH) is an iron overload disorder resulting from defective release or activity of hepcidin [1], the hepatic hormone that inhibits iron absorption by binding to and inactivating ferroportin [2], the only known cellular iron exporter involved in duodenal iron absorption and iron recycling from macrophages. HH is most frequently related to homozygosity for the p.C282Y mutation in the *HFE* gene ($C282Y^{+/+}$) [3], which hampers hepcidin upregulation by iron stores determining an increase in serum iron, which leads to its progressive accumulation in the liver and other parenchymal organs. However, the phenotypic expression is unpredictable and highly variable, as iron overload does not develop in all $C282Y^{+/+}$ individuals and not always leads to hepatic fibrosis [4,5], cirrhosis, and hepatocellular carcinoma (HCC), the most feared clinical complications of the disease [6,7].

Most of the $C282Y^{+/+}$ male subjects develop expanded iron stores during life, while due to the physiological iron losses during fertile age, and possibly also to a lower threshold for hepcidin release [8], female gender represents a major protective factor. In population-based screening studies, it has been shown that 75–94% of $C282Y^{+/+}$ males develop elevated transferrin saturation (TS), and that 64–68% will have an increased serum ferritin [4,9–12]. However, even in males, the prediction of clinical disease remains uncertain [13].

The recognition of the incomplete penetrance of HH has led to a search of modifiers of clinical expression, including alcohol intake and hepatitis virus infection [14,15], and genetic factors, such as the beta-thalassemia trait and polymorphisms influencing hepcidin release, inflammation and liver damage, and the response to oxidative stress [13,16–23].

The transmembrane protease serine 6 (*TMPRSS6*) gene encodes type 2 transmembrane serine protease matriptase-2, which cleaves the membrane-bound hemojuvelin, a bone morphogenetic protein (BMP) co-receptor required for hepcidin expression in the liver, thereby decreasing hepcidin transcription [24]. TMPRSS6 is induced by iron via BMP-6, thus providing a negative feedback for iron release during iron overload [25]. Rare loss-of-function germline mutations in *TMPRSS6* cause iron-refractory iron deficiency anemia (IRIDA) by upregulating hepcidin expression due to the inability to cleave hemojuvelin [26],

whereas common *TMPRSS6* polymorphisms have been shown in genome-wide association studies to represent a major determinant of iron-restricted erythropoiesis. The strongest *TMPRSS6* association was observed for rs855791 C >T polymorphism in exon 17, a non-synonymous substitution encoding the p.A736V variant that reduces the ability of the enzyme to cleave hemojuvelin. The p.736V allele was associated with lower serum iron and TS, decreased hemoglobin [8,26–29], and a less efficient *in vitro* inhibition of hepcidin transcription compared to the 736A allele [30]. In addition, the rs855791 polymorphism seems to be differentially represented in alternative TMPRSS6 transcripts, as the T allele (Val) is present in most RNAs (6 of 7 having exon 17), whereas only the AY358398 transcript, which seems to be driven by a different promoter, displays the C allele (Ala).

Importantly, *TMPRSS6* disruption influences iron metabolism and expression of hemochromatosis in the $Hfe^{-/-}$ mouse model [31,32], as well as in a model of beta-thalassemia [33], but there are still no data available on the effect of *TMPRSS6* polymorphisms on HH expression.

The aim of this study was therefore to assess the influence of the A736V *TMPRSS6* variant on the penetrance and clinical expression of HH in a large series of well-characterized Italian patients.

Patients and methods

Patients

We retrospectively considered 315 consecutive unrelated patients of Italian ancestry with phenotypically-expressed HH (i.e., with TS and serum ferritin above the upper normal values, confirmed at least in two different occasions) from two centers in Northern Italy (Milan area) included in a previous study on the natural history of the disease [7], for whom DNA samples were available. The *HFE* genotype was $C282Y^{+/+}$ in 163 cases; 70 of the remaining 152 patients were positive for C282Y/H63D, $H63D^{+/+}$ or C282Y/wild type (wt) *HFE* genotypes, whereas 82 patients were negative for both mutations or were H63D/wt. Of this last group of patients, four had established genetic determinants of iron overload (hemojuvelin, ferroportin-1, transferrin receptor-2, and hepcidin mutations). Only a few other patients were found to be affected by possible hereditary risk factors for iron overload: one was a heterozygous carrier of the E302K hemojuvelin mutation, and two were positive for the 72C >T hepcidin promoter mutation. The study flow chart is presented in Fig. 1.

The diagnosis of HH was based on clinical, biochemical, and/or histological evidence of the hemochromatosis phenotype as defined by classical diagnostic criteria [7,34,35], including hepatic iron concentration, TS percentage, serum ferritin, and iron removed to reach depletion, which was estimated as previously reported [36].

Complete medical history, routine biochemical and serological data, liver ultrasonography, and physical examination reports at diagnosis were available in all cases and evaluated at the time of diagnosis, as well as a careful estimation of both daily alcohol consumption and smoking habits in the previous 5 years, which was confirmed by at least one family member [7]. Alcohol abuse was defined as a daily alcohol intake >50 g in men and >40 g in women for more than 5 years. Chronic viral hepatitis was systematically searched for in all subjects evaluated by means of HBsAg and anti-HCV antibodies determination.

Diagnosis of cirrhosis was based upon liver histology or clinical evidence (in patients with hepatic decompensation or portal hypertension, in whom liver biopsy was not indicated for ethical reasons), and on clinical criteria in the remaining cases (when liver biopsy was not indicated according to current guidelines) [37]. Cirrhosis was considered clinically absent only if all these conditions were satisfied: (a) age <40 years, (b) ALT within normal levels, (c) and ferritin <1000 ng/ml. These criteria have been shown to rule out advanced fibrosis with high specificity in patients with $C282Y^{+/+}$ HH without viral hepatitis and excessive alcohol intake [37]. HH-related non-hepatic organ involvement was evaluated as previously described in all patients presenting with ferritin levels $\geqslant$1000 ng/ml and when clinically indicated [16,38,39].

Tissue sections were stained with hematoxylin and eosin, impregnated with silver for reticulin framework, and stained with trichrome for collagen and Perls for iron. Fibrosis was scored according to Ishak [40]. The minimum biopsy size was 1.7 cm and the number of portal areas 10.

Patients were submitted to an iron-depleting regimen by weekly phlebotomy up to iron depletion, and thereafter to maintenance phlebotomy [7]. HCC was diagnosed according to the American Association for the Study of the Liver (AASLD) guidelines [41], and age at diagnosis was recorded. The cumulative incidence of HCC was assessed on June 1st, 2011. Data were censored at the last outpatient visit.

We considered 271 healthy blood donors with normal iron parameters (serum ferritin and TS at first blood donation) as controls, all of Italian ancestry from Northern Italy (Milan area). Clinical features of patients and controls are shown in Table 1. Written informed consent was obtained from each subject. The study conforms to the ethical guidelines of the 1975 declaration of Helsinki and was approved by the Institutional Review Board of the Fondazione IRCCS Ca' Granda.

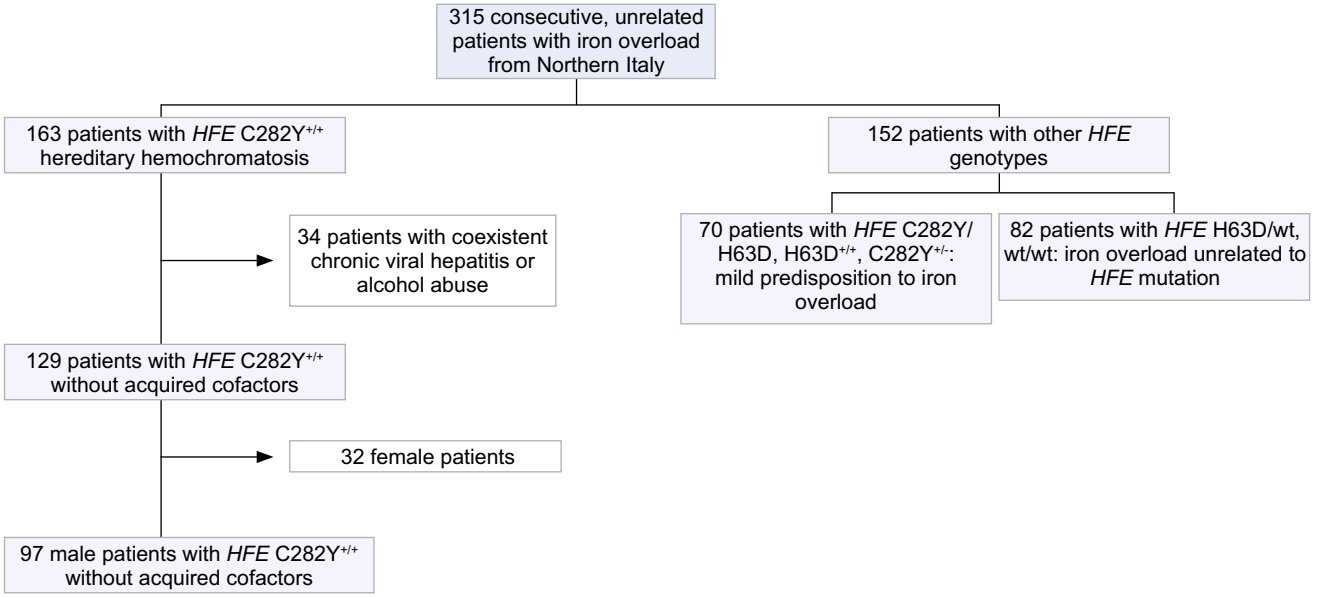

Fig. 1. Study flow chart.