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Hepcidin and iron-related gene expression in subjects with Dysmetabolic Hepatic Iron Overload ☆

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***Background/Aims*: Many patients with hepatic iron overload do not have identifiable mutations and often present with metabolic disorders and hepatic steatosis. Since the pathophysiology of Dysmetabolic Hepatic Iron Overload (DHIO) is still obscure, the aim of this study was to evaluate, in these patients, possible alterations in iron-related molecule expression.**

***Methods*: Iron-related gene mRNA levels were determined by quantitative-PCR in liver biopsies of subjects with NAFLD without iron overload and patients with HFE-hemochromatosis, β-thalassemia major and DHIO. Urinary hepcidin was measured by immunoblotting.**

***Results*: No alterations in mRNA expression of either iron transporters or exporters were found in DHIO. mRNA and urinary hepcidin levels normalized for the amount of iron overload showed a significantly lower ratio than in controls, although not as low as in hemochromatosis or β-thalassemia. Differently from what observed in hemochromatosis, hepcidin mRNA did not correlate with urinary hepcidin.**

***Conclusions*: Patients with DHIO show appropriate regulation of mRNAs encoding proteins involved in iron uptake and efflux but dysregulation of hepcidin production. The relatively elevated urinary hepcidin can explain the iron phenotype in DHIO (more macrophage iron retention and low/normal transferrin saturation).**

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***Keywords*: Iron overload; Hepcidin; Hemochromatosis; Transferrin Receptor; Gene expression; Quantitative-PCR**

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Abbreviations: DHIO, Dysmetabolic Hepatic Iron Overload; TfR, Transferrin Receptor; BMP, bone morphogenetic protein; NAFLD, Non-Alcoholic Fatty Liver Disease; HFE-HH, HFE-hemochromatosis; FP, ferroportin; HIS, Hepatocyte Iron Score; SIS, Sinusoidal Iron Score; PIS, Portal Iron Score; TIS, Total Iron Score.

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1. Introduction

The discovery of key new genes and proteins has led to the molecular characterization of forms of primary iron overload caused by mutations of *HFE* [1], *hemojuvelin* and *HAMP* (*hepcidin*) [2,3] *Transferrin Receptor 2* (*TfR2*) [4] and *ferroportin* (*FP*) [5]. Apart from rare mutations in *ceruloplasmin* [6] and H-*ferritin* [7], there remain conditions in which iron overload cannot be explained by known genetic defects and the pathophysiology of iron accumulation is still obscure. Increased liver iron deposits have been reported in patients with Non-Alcoholic Fatty Liver Disease (NAFLD), one of the most common forms of chronic liver disease [8,9]. Approximately 30% of NAFLD patients have elevated ferritin and a specific form of iron overload has been described and variably named Insulin Resistance- or Dysmetabolic-Hepatic Iron Overload syndrome [10,11].

In these conditions the liver represents the major site of iron deposition and has a central role in iron homeostasis through the regulation of hepcidin production. [12–14]. Hepcidin, the principal iron regulatory hormone, controls plasma iron levels by blocking intestinal iron absorption and iron recycling by macrophages. Hepcidin binds to ferroportin, the only known iron exporter, and induces its internalization and intracellular degradation [15–17]. Hepatic hepcidin production is modulated by different influences: circulating iron and iron stores, erythropoietic activity, hypoxia and inflammatory stimuli that act through pathways involving IL-6, other cytokines, and bone morphogenetic proteins (BMPs) [18–26].

Studies in human subjects and in animal models indicate that most hereditary hemochromatoses are associated with the inadequate production or deficiency of hepcidin, resulting from mutations in the hepcidin gene itself or in hepcidin regulators, *HFE*, *TFR2* and hemojuvelin (*HJV*) [27–32]. HFE and TfR2 may interact and function as an iron sensor [33], whereas hemojuvelin acts as a BMP co-receptor and likely regulates hepcidin transcription through the BMP/SMAD pathway [25].

In contrast to hereditary hemochromatoses, little is known about the involvement of these regulators in Dysmetabolic Hepatic Iron Overload (DHIO), such as those associated with disorders of glucose and lipid metabolism. The aim of this study was thus to describe the expression of hepcidin and other iron-related molecules in patients with DHIO.

2. Materials and methods

2.1. Patient selection

Forty-nine patients, 10 with HFE-hemochromatosis (HFE-HH), 25 with DHIO, 6 with β-thalassemia major and 8 with non-alcoholic fatty liver disease (NAFLD) without iron overload were enrolled in the study. Patients with HFE-hemochromatosis were untreated C282Y homozygotes that underwent liver biopsy for diagnostic purposes (serum ferritin level > 1000 μg/L). Patients with DHIO presented with increased serum ferritin levels, hepatic iron overload at liver biopsy, with or without increased transferrin saturation. Exclusion criteria for this group were hemochromatosis type I, II, III and IV, iron-loading anemias and history of blood transfusions or treatment with parenteral iron, acute and chronic inflammatory disorders, chronic viral or autoimmune hepatitis, end-stage liver disease, porphyria cutanea tarda, aceruloplasminemia, alcohol intake >40 g/day and >20 g/day, in men and women, respectively. None of the DHIO patients was heterozygous for the C282Y mutation, and 2 were heterozygous for the H63D mutation. All DHIO patients had at least one feature of the metabolic syndrome according to the US National Cholesterol Education Program ATPIII [34]. Twenty-one (84%) had also NAFLD defined as the presence of at least 5% steatosis in liver biopsy [35]. Patients with β-thalassemia major underwent liver biopsy at least 15 days after the last transfusion to assess the degree of liver damage and iron overload. Patients with NAFLD without hepatic iron overload (Deugnier score < 6), and absent or minimal fibrosis and inflammation were regarded as controls. None of these patients had HBV or HCV infection, autoimmune hepatitis, α1 antitrypsin deficiency or Wilson's disease.

All patients had given written informed consent to genetic testing analysis and liver biopsy before enrolment in the study. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a *priori* approval by the Ethical Committee of the San Gerardo Hospital.

2.2. DNA extraction and genotyping

Genomic DNA was extracted from peripheral blood leukocytes using the Wizard genomic DNA purification kit (Promega, Madison, WI, USA), according to the manufacturer's instructions. All the primers employed for sequencing the *HFE, FP, TFR2, HAMP* genes were previously reported by Lee et al. [36] and for *HFE2* by Lanzara et al. [37]. Sequencing was performed using the ABI Prism terminator cycle sequencing ready reaction kit on an ABI Prism 3100 Avant DNA sequencer (PE Applied Biosystems, Foster City, CA, USA). Every sequence was compared with their corresponding reference sequence available in GeneBank (GenBank sequence Accession Nos. NC_000002.10, NC_000006.10, NC_000001.9, NC_000019.8, and NC_000007.12, for *SLC40A1*, *HFE, HFE2*, *HAMP*, and *TFR2* genes, respectively).

2.3. Liver biopsy processing

Liver biopsy was performed for diagnostic purposes, after obtaining the informed consent. Immediately after the procedure, the biopsy was cut into two pieces. A 2.5 cm piece was fixed in 10% formalin (pH 7.4) for histology, whereas a 0.5–1 cm of tissue from needle liver biopsy was snap-frozen in liquid nitrogen for mRNA analysis. Hepatic sections were stained with HE and Perls staining for routine histology and iron deposition, respectively. Grading and staging of liver damage were performed as described by Ishak [38]. Liver iron overload was assessed as described by Deugnier et al. [39], with Hepatocyte Iron Score (HIS) ranging from 0 to 36, Sinusoidal Iron Score (SIS) from 0 to 12, and Portal Iron Score (PIS) from 0 to 12. The sum of HIS, SIS and PIS is the Total Iron Score (TIS) which ranges from 0 to 60.

2.4. Urinary hepcidin measurement

Urinary hepcidin concentration was assessed at the time of liver biopsy in 8 subjects with NAFLD, 20 with DHIO and 8 HFE-HH. After collection, urines were preserved with 0.05% sodium azide, and stored at −80 °C until measurement. Urinary hepcidin immunodot assay was performed as previously described [19]. Urinary hepcidin was normalized to urinary creatinine concentration (ng hepcidin/mg creatinine). To relate hepcidin levels to the degree of iron loading in each patient, the hepcidin–ferritin ratio was calculated as previously described [30].

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