Research Article

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Ferroportin disease: A systematic meta-analysis of clinical and molecular findings☆

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Background & Aims: *Classical* ferroportin disease is characterized by hyperferritinemia, normal transferrin saturation, and iron overload in macrophages. A *non-classical* form is characterized by additional hepatocellular iron deposits and a high transferrin saturation. Both forms demonstrate autosomal dominant transmission and are associated with ferroportin gene (*SLC40A1*) mutations. *SLC40A1* encodes a cellular iron exporter expressed in macrophages, enterocytes, and hepatocytes. The aim of the analysis is to determine the penetrance of *SLC40A1* mutations and to evaluate *in silico* tools to predict the functional impairment of ferroportin mutations as an alternative to *in vitro* studies.
Methods: We conducted a systematic review of the literature and meta-analysis of the biochemical presentation, genetics, and pathology of ferroportin disease.
Results: Of the 176 individuals reported with *SLC40A1* mutations, 80 were classified as *classical* phenotype with hyperferritinemia and normal transferrin saturation. The *non-classical* phenotype with hyperferritinemia and elevated transferrin saturation was present in 53 patients. The remaining patients had normal serum ferritin or the data were reported incompletely. Despite an increased hepatic iron concentration in all biopsied patients, significant fibrosis or cirrhosis was present in only 11%. Hyperferritinemia was present in 86% of individuals with ferroportin mutations. Bio-informatic analysis of ferroportin mutations showed that the PolyPhen score has a sensitivity of 99% and a specificity of 67% for the discrimination between ferroportin mutations and polymorphisms.
Conclusions: In contrast to HFE hemochromatosis, ferroportin disease has a high penetrance, is genetically heterogeneous and is rarely associated with fibrosis. *Non-classical* ferroportin disease is associated with a higher risk of fibrosis and a more severe overload of hepatic iron.

Keywords: SLC40A1; Hemochromatosis type IV; Iron; Bio-informatics; SLC11A3; IREG1; FPN1; PolyPhen.
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Abbreviations: AUROC, area under the receiver operator curve; ER, endoplasmic reticulum; HIC, hepatic iron concentration; JAK2, Janus Kinase 2; MRI, magnetic resonance imaging; PolyPhen, polymorphism phenotyping; ROC, receiver operator curve; SIFT, sorting intolerant from tolerant.

Introduction

Ferroportin disease is a clinically and genetically heterogeneous iron overload syndrome [1]. Its clinical presentation has been documented in individual case reports and small case series. The natural course has been reported in one small series [2]. Hyperferritinemia, a normal to low transferrin saturation and Kupffer cell iron storage, presenting as hepatic and spleen iron overload, are considered characteristic features of *classical* ferroportin disease [3]. Increased transferrin saturation and hepatocellular iron overload, in addition to hyperferritinemia and macrophage iron loading, is considered characteristic for the *non-classical* phenotype [3,4].

A genotype–phenotype correlation was suggested to explain the clinical heterogeneity of the disease where most mutations (e.g. A77D, D157G, V162del, N174I, Q182H, Q248H, and G323V) are associated with *classical* ferroportin disease [5–8]. Distinct mutations (e.g. N144H, Y64N, C326Y/S, S338R, Y501C) have been found in patients who presented with the *non-classical* phenotype [5,9–12].

Ferroportin is the only known mammalian iron exporter and is expressed in macrophages and the basolateral membrane of enterocytes and hepatocytes [13–15]. In *classical* ferroportin disease, macrophage iron overload results from cellular iron export deficiency, i.e. loss of ferroportin function [6]. The D157G mutation leads to hepcidin-independent, constitutive binding of Janus Kinase 2 (JAK2) and thus triggers ferroportin down-regulation [16]. The concept that other loss of function mutations of ferroportin result in intracellular retention of the iron export pump has been challenged recently [17,18], but alternative mechanisms leading to iron transport deficiency have not been fully elucidated.

Ferroportin inactivation is mediated by the peptide hormone hepcidin [19,20]. Distinct mutations render ferroportin resistant

to inactivation by hepcidin and cause a gain of iron export function, which results in hyper-absorption of dietary iron.

Hepcidin is secreted by hepatocytes into the circulation in response to pro-inflammatory cytokines, hepatocellular iron loading, and endoplasmic reticulum stress [21,22]. Circulating hepcidin directly interacts with ferroportin and induces its internalization and degradation [23]. The free thiol group of cysteine 326 of ferroportin is essential for hepcidin binding and ferroportin gene mutations affecting residue 326 result in hepcidin resistance [7,24]. Patients with ferroportin disease due to hepcidin resistance mutations, exhibit a *non-classical* phenotype [8,25,26].

Functional studies in cells overexpressing ferroportin variants have been used to differentiate between gain-of-function and loss-of-function mutations and benign sequence variants. The functional relevance of ferroportin mutations can also be deduced from mutation segregation studies within families with ferroportin disease. Association studies of *SLC40A1* polymorphisms with serum iron indices in population studies indirectly provide evidence for their functional relevance [27–29].

Bio-informatic tools were used to complement the genetic and functional studies. SIFT [30] and PolyPhen [31] provide an *in silico* prediction of the functional consequences of missense mutations. The use of these tools in differentiating between diseases-associated ferroportin mutations from benign sequence variants was assessed. The clinical presentation and penetrance of ferroportin gene mutations were assessed from a systematic review of the literature.

Methods

Search strategy

A systematic literature search was undertaken in Medline from 1996 to June 2009 according to the following search strategy: #1 ('*SLC40A1*' or 'SLC11A3' or 'ferroportin' or 'IREG' or 'IREG1' or 'IREG-1' or 'ferroportin' or 'ferroportin1' or 'ferroportin-1' or 'FPN' or 'FPN1' or 'FPN-1').mp. [mp = title, original title, abstract, name of substance word, subject heading word]; #2 ('mutation' or 'variant').mp. [mp = title, original title, abstract, name of substance word, subject heading word]; # 3: #1 and #2. This search retrieved 143 references and was supplemented by manual searching for references of the relevant articles identified. After exclusion of review articles and non-human studies, data from 49 studies were included. This search was complemented by an Embase search using the search strategy: #1 (?*SLC40A1*? or ?SLC11A3? or ?ferroportin? or ?IREG? or ?IREG1? or ?IREG-1? or ?ferroportin? or ?ferroportin1? or ?ferroportin-1? or ?FPN? or ?FPN1? or ?FPN-1?).mp. [mp = title, original title, abstract, name of substance word, subject heading word]; #2 (?mutation? or ?variant?).mp. [mp = title, original title, abstract, name of substance word, subject heading word]; # 3: #1 and #2.

Prediction of the functional effect of amino acid substitutions by in silico analysis

Two algorithms were used to evaluate the impact of amino acid substitutions on the function of ferroportin: polymorphism phenotyping (PolyPhen) [31–33] and sorting intolerant from tolerant (SIFT) [30]. Genetic variants of SLC40A1, identified in patients with suspected iron overload, were collected from the systematic review of the literature. In addition, single nucleotide polymorphisms of SLC40A1 were collected from the literature and from ENSEMBL (http://www.ensembl.org/) and the NCBI databases (http://www.ncbi.nlm.nih.gov/). A SLC40A1 variant was considered a mutation in case its frequency in the control population was undetermined, or lower than 1:50, and the variant was not reported in a SNP database [27–29,34,35].

PolyPhen

PolyPhen (= *Poly*morphism *Phen*otyping) is an automatic tool for prediction of possible impact of an amino acid substitution on the structure and function of a human protein. The predictive value of the PolyPhen score was demonstrated in *diabetes mellitus* [36] and cancer genetics [37]. This prediction is based on empirical rules which are applied to the sequence, phylogenetic, and structural information characterizing the substitution [31]. PolyPhen was employed, as available at http://coot.embl.de/PolyPhen/ (version August 12, 2008), using the NCBI protein accession number Q9NP59 (*SLC40A1*) for ferroportin. In all cases, the prediction basis was only the default position-specific independent counts (PSIC) score derived from multiple sequence alignment of observations. PolyPhen scores of >2.0 indicated protein function as 'probably damaging' and scores of <1.5 as 'benign'. A PolyPhen score of 1.5–2.0 is classified as 'possibly' damaging.

A phylogenetic sequence alignment of selected ferroportin protein sequences is shown in Fig. 2.

SIFT

SIFT (= Sorting Intolerant from Tolerant) is a sequence-homology-based tool that presumes that important amino acids will be conserved in the protein family. Changes at well-conserved positions tend to be predicted as deleterious. SIFT is a multi-step procedure that uses multiple alignment information to predict tolerated and deleterious substitutions for every position of the query sequence [30]. The SIFT Blink algorithm was applied to compare amino acid sequences of different organisms chosen from precomputed NCBI BLAST searches. For query in SIFT Blink, GI: 49065554 (*SLC40A1*) was used to analyze the protein sequence by using the parameter 'best BLAST hit to each organism' and omitting sequences >90% identical to query. Edited alignments were reanalyzed by the appropriate module of SIFT (http://blocks.fhcrc.org/sift/SIFT_aligned_seqs_submit.html). Results were reported as 'affects protein function' or 'tolerated' according to this analysis.

To evaluate the bio-informatic tools to distinguish between benign and disease sequence variants, the SLC40A1 polymorphisms were used as true negatives and genetic variants of SLC40A1 identified in patients with ferroportin disease as true positives. The PolyPhen and SIFT scores were then analyzed in a ROC curve for their sensitivity and specificity.

Statistical analysis

For statistical analysis the software package for social sciences (SPSS v15.0) was used. To test for correlation between parameters, Pearson correlation was carried out for normally distributed parameters and Spearman rank correlation for non-normally distributed parameters. Normality of distribution was tested by Kolmogorov–Smirnov curve-fitting. Differences between groups were analyzed by Kruskal Wallis test for non-Gaussian distributed variables. Correlations and differences were considered significant if $p < 0.05$. Sensitivity and specificity of SIFT and PolyPhen scores were calculated form the ROC curve analysis using mutations identified in patients with ferroportin disease as true positives and polymorphisms reported in the SNP database (http://www.ncbi.nlm.nih.gov/sites/entrez) as true negatives.

Results

High biochemical penetrance and variable clinical presentation of SLC40A1 mutations

One hundred and seventy-six individuals with ferroportin gene mutations were reported in the literature, and their demographic and biochemical data are listed in Table 1. The diagnosis of ferroportin disease was most frequently made in the 3rd to 4th decade of life and 43% of patients were female.

To describe the disease presentation and the penetrance of underlying mutations, serum iron parameters, liver biopsy findings, MRI, comorbidities, and phlebotomy status were assessed. These were reported incompletely for several patients (Table 1).

Forty-five comorbidities were reported in 29 patients (viral hepatitis 3, steatosis 12, obesity 5, diabetes 8, impaired glucose tolerance 2, hyperlipidemia 2, increased alcohol consumption 2, and sarcoidosis 2). Mean age and mean serum ferritin were significantly higher in patients with comorbidities, than in patients without comorbidities, (52 ± 15 years vs. 39 ± 20 years $p = 0.002$ and 3133 ± 3270 μg/L vs. 1965 ± 2067 μg/L, $p = 0.017$). Fibrosis $\geqslant$F1 (Metavir) was significantly more common in patients with