# BRIEF REPORTS

# Expression of hepcidin and other iron-related genes in type 3 hemochromatosis due to a novel mutation in transferrin receptor-2

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## ABSTRACT

Transferrin receptor-2 (*TFR2*) regulates hepatic hepcidin secretion and when mutated causes type-3 hemochromatosis. No functional study is available in humans. We studied a 47 year-old woman with hemochromatosis. *TFR2* DNA and its hepatic transcript were directly sequenced. Hepatic expression of hepcidin and other iron-related genes were measured by qRT-PCR. Urinary hepcidin was measured at baseline and after an oral iron challenge (ferrous sulfate, 65 mg) by SELDI-TOF-MS. A novel homozygous *TFR2* mutation was identified in the splicing donor site of intron 4 (c.614+4 A>G) causing exon 4 skipping. Hepcidin and hemojuvelin expression were markedly reduced. Urinary hepcidin was lower than normal and further decreased after iron challenge. This is the first description of iron-related gene expression profiles in a *TFR2* mutated patient. The decreased hepatic and urinary expression of hepcidin and lack of acute response to iron challenge confirms the primary role of TFR2 in iron homeostasis.

Key words: TFR2, hepcidin, hepatic expression, iron overload, splicing mutation.

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## Introduction

Homozygous mutations of Transferrin Receptor-2 (*TFR2*) have been linked to type 3 hemochromatosis (HH).[1] *TFR2* mutant or knockout mice[2-4] confirmed human findings indicating that TFR2 plays an important role in iron metabolism.

Although TFR2 is also able to bind transferrin-Fe(III) with a lower affinity than Transferrin Receptor-1, iron delivery to cells is not its primary function.[5] Diferric transferrin levels increase the stability of TFR2 protein that may act as a sensor of serum iron levels modulating iron absorption through hepcidin induction.[5-7] Hepcidin is the main iron regulator that functions to lower plasma iron levels by binding, internalizing and degrading the iron exporter ferroportin.[5] Hepcidin production is also modulated by other influences: iron stores, erythropoietic activity, hypoxia and inflammation that act through BMPs/SMAD4 pathways.[5,8]

Patients with type-3 HH showed low urinary hepcidin levels[9] and *TFR2* mutant mice did not up-regulate hepatic hepcidin expression in response to iron loading.[2-4] Most of our knowledge of the contribution of TFR2 to iron homeostasis derives from studies of complete or liver specific *TFR2*-knockout (KO) mice.[3,4] These studies demonstrated that the liver is the primary site for *TFR2* expression and activity and that liver-expressed *TFR2* is required for the regulation of hepcidin. No functional studies are presently available in humans. Here we report a novel *TFR2* mutation causing type-3 HH in a woman and we describe the hepatic expression of hepcidin and other iron-related molecules. We also measured urinary hepcidin excretion at baseline and after an oral iron challenge. We hypothesized that lack of a functional TFR2 may decrease or abolish urinary hepcidin excretion in response to acute iron challenge.

## Design and Methods

Genomic DNA was extracted from peripheral blood leukocytes and amplified by PCR using already designed primers.[10] PCR products were directly sequenced by ABI Prism 3100 Avant DNA sequencer (PE Applied Biosystems, Foster City, CA, USA) and compared with reference sequence (GenBank Accession N° NC_000007.12).

Liver biopsy was processed for histology and mRNA analysis. Assessment of hepatic fibrosis and iron overload, mRNA extraction and cDNA synthesis were performed as previously described in detail.[11] cDNA was stored at -80°C until its use. cDNA was amplified by PCR (*Online Supplementary Table S1*), directly sequenced and compared with reference sequence (GenBank Accession N° NM_003227.2).

mRNA expression levels of *BMP2, BMP4, CP, DMT1, HAMP, HFE, HJV, IL-6, SLC40A1, TFR1, TFR2* (the commercial probe was designed in exon boundary 3-4, Part Number Hs00162690_m1) and *HPRT1*, chosen as housekeeping gene, were evaluated by quantitative Real-Time PCR (qRT-PCR) as previously described.[11] The proband's results were compared to those observed in 10 patients HFE-HH (C282Y homozygotes) and 8 controls with non-alcoholic-fatty-liver (NAFLD) without hepatic iron overload, absent fibrosis and inflammation. Full clinical data of HFE-HH patients and NAFLD subjects are reported in Barisani *et al*.[11]

Urinary hepcidin was measured by SELDI-TOF-MS according to Bozzini *et al*.[12] A challenge test by 65 mg oral iron as ferrous sulfate (iron, 65 mg, CVS Pharmacy) was conducted to assess hepatic response to iron, as previously reported.[13]

Written informed consent for genetic study, liver biopsy and iron challenge were obtained according to the Institution's guidelines. 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 San Gerardo Hospital.

## Results and Discussion

### Case description

A 47 year-old woman came to our attention in January 2007. In 1998, serum ferritin and transferrin saturation were 2207 μg/L and 88% respectively. *HFE* (C282Y and H63D), *TFR2* (E60X, M172K, Y250X, AVAQ594-597 del) and Ferroportin1 (N144H, V162 del) mutations were absent. Hepatitis B and C viral infections, hematologic and inflammatory diseases were excluded. No further analysis and treatment were performed. At our evaluation, serum ferritin was 2674 μg/L, transferrin saturation 88%, hemoglobin 14.6 g/dL and mean corpuscular volume 89 fl. Both parents originated from the same village in Southern Italy but there was neither family history of consanguinity or hemocromatosis; her 2 offspring and 2 siblings have normal serum iron indices. She had regular menstrual blood losses, no history of alcohol intake, blood transfusions or parenteral iron administration. Metabolic syndrome, characterized by glucose intolerance, arterial hypertension, obesity and hypertriglyceridemia was present. Excluding a mild increase of serum alanine-aminotransferase (43 IU/L), no other alteration was found. Mild left ventricular hypertrophy, moderate hepato-splenomegaly without signs of portal hypertension were present. At biopsy, liver cirrhosis and moderate steatosis were present. A severe iron overload (Deugnier's total iron score: 42) with the typical hemochromatosis pattern was found.[14] The hepatic iron concentration, measured by quantitative MRI according to
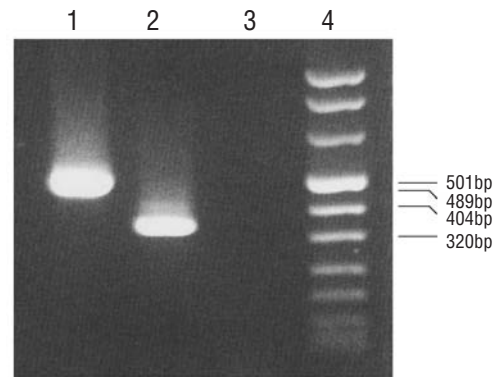


**Figure 1.** Electrophoresis of PCR products of *TFR2* cDNA amplicon 2 from proband and control. Lane 1: control; lane 2: proband; lane 3: negative; lane 4: DNA molecular Marker VIII (Roche Diagnostics GmbH, Mannheim, Germany).

Gandon *et al*.,[15] was >300 μmol/gr. No iron accumulation was found in the spleen. She began weekly phlebotomy treatment.

### DNA sequence analysis

The genomic sequence of *TFR2* revealed an A into G transition in the homozygous state in the splicing donor site, 4 nucleotides after exon 4 (c.614+4 A>G). The same mutation was found in the heterozygous state in the daughter and in one sibling. The mutation was not found in 50 healthy Italian controls.

### RNA analysis

The PCR product of the cDNA revealed an aberrantly shifted band (Figure 1) in the amplicon 2. Direct sequencing showed a deletion of 141 nucleotides caused by the skipping of the entire exon 4 without frameshift (Figure 2) as predicted by *in silico* studies (*http://www.cbs.dtu.dk/services/NetGene2* and *https://splice.cmh.edu/cgi-bin*). qRT-PCR revealed no TFR2 transcript in the proband since the commercial probe available is designed in exon boundary 3-4.

### Hepatic mRNA expression

The proband's hepatic mRNA level of the genes analyzed is reported in Table 1 together with those measured in *HFE*-HH and controls.

### Urine hepcidin measurement

At baseline, urinary hepcidin was 0.060 MIU/mM creatinine (normal values 0.49 MIU/mM creatinine, 95% CIs 0.31 to 0.66),[12] and 0.035 MIU/mM creatinine 24 hours after the oral iron challenge.

We describe a novel *TFR2* mutation in a patient with type-3 HH and, for the first time, hepatic expression profiles of hepcidin and iron-related genes. We demonstrate that this mutation causes a decreased urinary hepcidin excretion, a reduced hepatic *HAMP* and *HJV* expression, and the lack of hepcidin response to an oral iron challenge.

The mutation causes the skipping of the entire exon 4 with no frameshift (Figure 2). This leads to an abnormal protein lacking 47 aminoacids (from R158 to P205) in the extracellular domain where most of *TFR2* mutations are located.[1] It was recently shown that *TFR2* mutations result
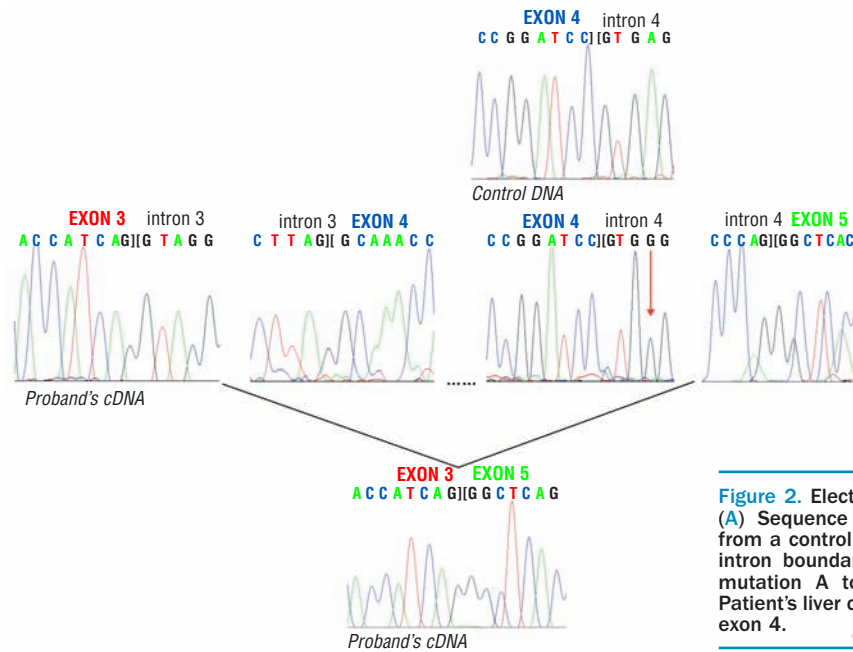
Figure 2. Electropherogram of the region of interest. (A) Sequence of exon-intron boundary 4 obtained from a control DNA. (B) Sequences of mutated exon-intron boundaries of exons 3,4 and 5 showing the mutation A to G (arrow) from patient's DNA. (C) Patient's liver cDNA sequence showing the skipping of exon 4.

in endoplasmic reticulum retention of the mutant protein that becomes unable to perform its putative function at cell surface.[16] The absence of surface expression of mutant TFR2 would hamper the interaction with diferric transferrin and the activation of the signaling pathway resulting in hepcidin induction. This likely occurs also in the present case in which the lack of exon 4 extensively disrupts the extracellular domain of the full transmembrane protein. The patient had, indeed, a severe phenotype characterized by a marked iron overload despite female gender, relatively young age and presence of the metabolic syndrome. At variance from that observed in women with *HFE*-HH, menstrual blood losses and pregnancies,[17] obesity and steatosis[18] had no protective effect on the proband's penetrance and expression confirming the severity of the mutation and the importance of TFR2 in iron homeostasis. We cannot exclude that hepatic steatosis might have contributed to the development of cirrhosis as reported in *HFE*-HH.[19] The analysis of hepatic expression of hepcidin and other iron-related genes in the patient, compared with that observed in controls and in *HFE*-HH patients revealed similarities with the other functional studies only available in animal models.[3,4] *TFR1* and *DMT1* hepatic mRNA levels were decreased in the proband probably due to reduced Iron Responsive Protein binding activity and hepatocellular iron overload. Hepcidin mRNA was reduced and largely inappropriate to the amount of iron overload. Accordingly, the patient's urinary hepcidin was markedly low confirming that the lack of TFR2 function in hepatocytes impairs hepcidin production and chronic hepcidin increase in response to iron stores. This finding, observed at baseline, enforces previous results showing low or undetectable urinary hepcidin levels in 8 out of 10 type-3 HH patients.[9] Most of those patients, however, were studied after or during iron depletion therapy which might decrease the stimulus for hepcidin production even at a time remote from phlebotomy in HH patients.[13] Interestingly, likely due to the mild hepatic inflammation

associated with metabolic syndrome and hepatic steatosis, hepatic mRNA expression of *IL-6* was higher than in control groups. Although this was not associated with inflammatory manifestations at systemic level, it may increase hepcidin production,[20] suggesting that patient's urinary and hepatic mRNA hepcidin levels might be even less than observed. In addition, we showed that urinary hepcidin did not change (it even decreased) after an oral iron challenge. In normal adults, hepcidin excretion increased 24 hours after the ingestion of a small dose (65 mg) of iron as

Table 1. Hepatic mRNA levels of iron-related genes in the proband and patients with *HFE* hemochromatosis and controls normalized to *HPRT1*.

| Genes | Proband | HFE hemochromatosis (n=10) | Controls (n=8) |
|---|---|---|---|
| *HFE* ($2^{-\Delta\Delta Ct}$) | 0.33±0.06 | 0.46±0.17 (0.34-0.58) | 0.43±0.12 (0.33-0.53) |
| *HJV* ($2^{-\Delta\Delta Ct}$) | 0.15±0.01 | 0.49±0.20 (0.34-0.63) | 0.72±0.27 (0.49-0.94) |
| *HAMP* ($2^{-\Delta\Delta Ct}$) | 13.57±0.97 | 22.19±12.98 (12.21-32.18) | 34.58±13.74 (23.09-46.07) |
| *TFR1* ($2^{-\Delta\Delta Ct}$) | 0.33±0.04 | 0.43±0.18 (0.28-0.57) | 1.01±0.60 (0.51-1.51) |
| *SLC40A1* ($2^{-\Delta\Delta Ct}$) | 0.42±0.04 | 0.73±0.27 (0.54-0.92) | 0.83±0.35 (0.54-1.12) |
| *IL-6* ($2^{-\Delta\Delta Ct}$) | 1.21±0.11 | 0.42±0.34 (0.17-0.66) | 0.31±0.16 (0.14-0.47) |
| *CP* ($2^{-\Delta\Delta Ct}$) | 0.66±0.07 | 1.01±1.39 (0.01-2.00) | 0.82±0.40 (0.39-1.24) |
| *DMT1* ($2^{-\Delta\Delta Ct}$) | 0.27±0.13 | 0.33±0.10 (0.25-0.41) | 0.55±0.14 (0.43-0.67) |
| *BMP2* ($2^{-\Delta\Delta Ct}$) | 0.24±0.01 | 0.56±0.14 (0.45-0.67) | 0.62±0.14 (0.49-0.75) |
| *BMP4* ($2^{-\Delta\Delta Ct}$) | 0.25±0.00 | 0.36±0.13 (0.25-0.46) | 0.40±0.03 (0.36-0.44) |

*All analyses were carried out in triplicate. Data are expressed as mean±SD. In HFE patients and controls 95% CI is also reported in brackets.*

ferrous sulfate, indicating a rapid hepatic response to iron.[13,20] By contrast, patients with *HFE*-HH have a decreased hepcidin response to oral iron.[13] The lack of response in the proband indicates that also a defective TFR2 abrogates the acute iron sensing of transferrin saturation. This finding is intriguing if we consider that TFR2 likely participates, together with HFE, to the putative iron signaling complex that modulates hepcidin transcription by sensing changes in diferric Tf concentration.[6,7,21] TFR2 functions upstream of SMAD4 in activating the *HAMP* promoter, perhaps through an independent signal or by affecting signal transduction by the BMP receptors.[5,8] Interestingly, *HJV* and, to a lesser extent, *BMP2* transcripts were also markedly reduced suggesting an interaction between TFR2 and HJV in the regulation of hepcidin expression. We cannot exclude that inflammatory changes, evidenced by *IL-6* increased expression, might be implicated in decreasing *HJV* transcription in the patient considering that hepatic *Rgmc* (the murine ortholog of *HJV*) expression markedly decreased six hours after LPS injection in mice.[22] However, the response of *HJV* to subclinical inflammation, as that observed in the proband, is unknown and we did not observe significant reduction of hepatic *HJV* mRNA expression in patients with dysmetabolic iron overload syndrome.[11] The present findings should be taken with caution since they were obtained from a single patient. Nevertheless, the reduced *HJV* expression observed in complete *TFR2*-KO[3] enforces our results that need to be confirmed in future studies. HJV (a BMP coreceptor) is an essential component of a receptor complex that requires the activities of HFE, TFR2, the BMP receptors and the ligands BMP2 and BMP4 to mediate hepcidin regulation by iron.[5,23] While the interaction between HJV and a subset of BMP ligands and receptors in regulating hepcidin expression has been recently clarified,[24] the downstream consequence of TFR2 activity and its relationship with the other protein involved in hepcidin regulation need to be clarified at the molecular level.

## Authorship and Disclosures

SP: conception and design; performance of data, analysis and interpretation; manuscript writing. RM: provision of study patients; analysis and interpretation; manuscript writing. PT: provision of study patients; analysis and interpretation; manuscript writing. SC: collection and assembly of data; manuscript writing. MP: collection and assembly of data. VP: collection and assembly of data. DB: analysis and interpretation; revision. AP: conception and design; data analysis and interpretation; manuscript writing; final approval of manuscript.

The authors report no potential conflicts of interest.

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