Hepcidin is decreased in TFR2 hemochromatosis

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The hepatic peptide hepcidin is the key regulator of iron metabolism in mammals. Recent evidence indicates that certain forms of hereditary hemochromatosis are caused by hepcidin deficiency. Juvenile hemochromatosis is associated with hepcidin or hemojuvelin mutations, and these patients have low or absent urinary hepcidin. Patients with C282Y HFE hemochromatosis also have inappropriately low hepcidin levels for the degree of iron loading. The relationship between the hemochromatosis due to transferrin receptor 2 *(TFR2)* mutations and hepcidin was unknown. We measured urinary hepcidin levels in 10 patients homozygous for *TFR2* mutations, all with increased transferrin saturation. Urinary hepcidin was low or undetectable in 8 of 10 cases irrespective of the previous phlebotomy treatments. The only 2 cases with normal hepcidin values had concomitant inflammatory conditions. Our data indicate that TFR2 is a modulator of hepcidin production in response to iron. (Blood. 2005;105:1803-1806)

Introduction

The hepatic peptide hepcidin is the central regulator of iron absorption in mammals. Evidence is accumulating that the pathophysiology of hemochromatosis, a genetic disorder characterized by deregulation of iron absorption, converges on hepcidin. Total hepcidin deficiency characterizes the severe iron overload of juvenile hemochromatosis, which rarely results from hepcidin-inactivating mutations[1] and more frequently from mutations of the *HJV* gene encoding hemojuvelin.[2-3] Patients with hemojuvelin-related hemochromatosis have low/undetectable urinary hepcidin levels,[2] suggesting that hemojuvelin protein is an important regulator of hepcidin expression. Hepcidin mRNA is also decreased or inappropriately low for the degree of iron overload both in Hfe-deficient or Hfe (845A/845A) (C282Y) mice[4] and in patients with HFE hemochromatosis,[5] implying that *HFE* is another modulator of hepcidin production in response to iron loading.

A rare form of hemochromatosis is due to mutations of transferrin receptor 2 *(TFR2),*[6] a member of the transferrin receptor family with an unclear function in iron metabolism. TFR2 has a capability of binding and internalizing diferric transferrin.[7] However, cellular iron uptake might not be the function of TFR2 in vivo, because mutational disruption of *TFR2,* both in humans[7] and in animal models,[8] leads to liver iron accumulation and not to iron restriction. In addition, iron overload that follows *TFR2* inactivation occurs early in life,[9] as in juvenile hemochromatosis, although TFR2-related disease runs a milder clinical course. Based on these observations, TFR2 could be another regulator of hepcidin, but its relationship with hepcidin in humans has so far remained speculative.

To ascertain the involvement of TFR2 in the hepcidin pathway, we measured urinary hepcidin levels in 10 hemochromatosis patients carrying different *TFR2* mutations. Our results show low/absent hepcidin in most patients, except for 2 who had concomitant inflammatory conditions. These results confirm the proposed role of TFR2 as a regulator of hepcidin production.

Patients, materials, and methods

Clinical data and molecular defects of the patients studied have been previously reported.[9-11] Controls were healthy adult subjects from the laboratory staff and their children. Informed consent was obtained from all subjects involved in the study or from parents in case of children, according to the guidelines of the different institutions. The study was approved by the Institutional Review Board of the Department of Clinical and Biological Sciences of the University of Turin, Italy.

Transferrin saturation and serum ferritin were measured by standard procedures. Urines of patients and controls were collected in Italy, preserved with 0.05% sodium azide, and shipped frozen to Los Angeles, California. Additional controls were obtained in Los Angeles. Urinary hepcidin assay was performed as previously described.[12] Cationic peptides were extracted from urine using CM Macro-prep (Bio-Rad Laboratories, Hercules, CA). Hepcidin concentrations were determined by an immunodot assay. Urine extracts equivalent to 0.1 to 0.5 mg creatinine were dotted on Immobilon-P membrane (Millipore, Bedford, MA) along with a range of synthetic hepcidin standards (0-80 ng). Hepcidin was detected using rabbit anti–human hepcidin antibody[12] with goat anti–rabbit horseradish peroxidase (HRP) as a secondary antibody. Dot blots were developed by the chemiluminescent detection method (SuperSignal West Pico Chemiluminescent Substrate; Pierce Chemical, Rockford, IL) and quantified with the Chemidoc cooled camera running Quantity One software (Bio-Rad Laboratories).

Hepcidin quantity in each sample was normalized using urinary creatinine concentrations measured in UCLA Clinical Laboratories, and

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Table 1. Characteristics of the patients studied

Sample no.	TFR2 genotype	Age, y	Sex	Hb level, g/dL	TS, %	Ferritin level, μg/L	Nanograms of hepcidin per milligram of creatinine	Therapy	Interval from last phlebotomy	Reference no.
1	AVAQ homozygote	37	M	15.7	105	33	< 1	Phlebotomy	15 d	14
2	AVAQ homozygote	35	M	14.7	100	193	7	Phlebotomy	15 d	14
3	M172K homozygote*	55	M	12	82	300	< 1	deferoxamine 1 g s.c. 2x weekly	NA	16
4	Y250X homozygote†	18	M	15.7	88	94	4	Phlebotomy	3 mo	12
5	Y250X homozygote	66	F	13.1	78	292	19	Phlebotomy	2 mo	13
6	Y250X homozygote	38	M	14.9	100	697	17	Phlebotomy	1 d	13
7a	Y250X homozygote†	3.5	M	11.9	70	45	57	—	NA	12
7b	Y250X homozygote†	3.5	M	ND	ND	ND	90	—	NA	
8	E60X homozygote	42	M	15.7	78	623	15	Phlebotomy	3 y	13
9	E60X homozygote	48	F	12.5	82	159	46	Phlebotomy	3 y	13
10	E60X homozygote	46	F	13.0	83	23	8	—	NA	13
11	HJV homozygote*	33	F	10.3	233	13	< 1	Phlebotomy	2 mo	3
12	AVAQ heterozygote	62	F	14.5	26	47	58	NA	NA	14
13	AVAQ heterozygote	67	M	16.8	34	66	82	NA	NA	14
14	AVAQ heterozygote	30	F	15.8	16	15	16	NA	NA	14
15	Control 1	23	M	16.6	ND	126	113	NA	NA	
16	Control 2	25	M	15.6	ND	98	105	NA	NA	
17	Control 3	3	M	11.4	ND	ND	49	NA	NA	
18	Control 4	5	F	12	ND	ND	80	NA	NA	

Normal values for adults are as follows: 20% to 40% TS, less than 200 μg/L ferritin for females and less than 300 μg/L ferritin for males, and 10 to 200 ng hepcidin per milligram of creatinine using the immunoassay described by Nemeth et al.[12]

Hb indicates hemoglobin; TS, transferrin saturation; phlebotomy, mantainance phlebotomy; ND, not done; NA, not applicable; and s.c., subcutaneously.

*β thalassemia trait.

†H63D heterozygote.

urinary hepcidin levels were expressed as nanograms of hepcidin per milligram of creatinine.

Results

Molecular and clinical data of all patients examined are reported in detail elsewhere.

Table 1 shows the list of the studied cases, their *TFR2* mutation status, and iron parameter levels at the time of urinary hepcidin measurements. Patients were homozygous for 4 different mutations (Y250X, E60X, M172K, and AVAQ 594-597del). Most had been previously treated by phlebotomy and were on maintenance therapy with 2 to 4 phlebotomies per year. As shown in Table 1, the interval from the last phlebotomy varied from 1 day to 3 years but in most cases was more than 15 days. Patient 3 had reduced phlebotomy tolerance because of mild anemia due to concomitant β-thalassemia trait and was treated with biweekly subcutaneous desferrioxamine injections.[13] Patient 10, previously reported as having iron deficiency,[10] never had phlebotomy and, at the time of the analysis, had ferritin in the low normal range. Serum ferritin levels were normal in most patients and increased in patients 5, 6, and 8 due to low compliance with treatment. All patients, however, had high transferrin saturation.

Five patients (cases 1 to 4 and 10) had hepcidin levels either undetectable or below the lower limit of the normal range, similar to the levels observed in juvenile hemochromatosis due to hemojuvelin or hepcidin mutations (Papanikolaou et al[2] and case 11) (normal range, 10-200 ng/mg creatinine, based on unrelated controls from the United States and cases 15 and 16). Cases 5, 6, and 8, who had suboptimal disease control, as indicated by their high serum ferritin, had hepcidin levels in the low end of the normal range (10-20 ng/mg creatinine) but inappropriate to the degree of iron loading. Two patients had hepcidin levels in the midnormal range (patients 7 and 9). Case 7, a 3-year-old untreated Y250X homozygote, had normal hepcidin levels in 2 different measurements. The child had a chronic oropharyngeal lymphoid hyperplasia and had frequent throat infections. The first urine sample was taken after an acute viral respiratory infection. A second sample, apparently taken after acute infection recovery, however, still showed normal values similar to those of age-matched controls (cases 17 and 18). The other patient with hepcidin values in the normal range (case 9) was healing from multiple bone fractures that occurred 6 months before the test.

Heterozygotes for *TFR2* mutations (cases 12 to 14) had urinary hepcidin in the normal range.

To relate the patients' hepcidin levels to the degree of iron loading, we calculated the hepcidin-ferritin ratio (Figure 1). All patients except case 7 had very low ratios as compared with the 3 *TFR2* heterozygotes and healthy controls.

Discussion

We report here that urinary hepcidin is low or undetectable in most patients with TFR2-related hemochromatosis. These findings indicate that TFR2 is a modulator of hepcidin production. Most patients had received phlebotomy treatment, but urinary hepcidin was measured in most cases after intervals of more than 15 days. It has been reported that phlebotomy suppresses hepcidin mRNA production in mice,[14] but in our experience hepcidin levels return to normal within 1 week after phlebotomy (Roetto et al, unpublished data, 2004). In addition, some patients were not fully iron depleted at the time of the study, and all had remarkably elevated transferrin saturation. Eight of 10

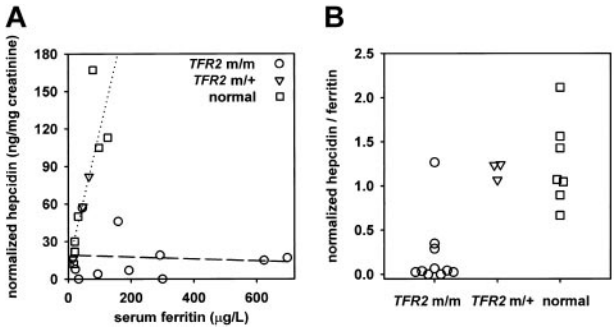

Figure 1. In patients with homozygous *TFR2* mutations, urinary hepcidin levels are inappropriately low relative to serum ferritin. Urinary hepcidin was normalized to urinary creatinine concentration (nanograms of hepcidin per milligram of creatinine). *TFR2* m/m (○) indicates patients homozygous for the *TFR2* mutations; *TFR2* m/+ (▽), subjects heterozygous for *TFR2* mutations. Healthy patients are indicated by □. (A) Relationship between normalized urinary hepcidin and serum ferritin. Linear regression in healthy subjects is represented by the dotted line and in *TFR2* homozygous patients by the dashed line. (B) Ratio of normalized urinary hepcidin to serum ferritin levels.

patients had hepcidin levels that are clearly inappropriately low for the degree of iron loading. Their hepcidin levels were either unmeasurable, below the lower normal limit (10 ng/mg creatinine), or in the low end of normal levels (10 to 20 ng/mg creatinine) (normal range, 10-200 ng/mg creatinine). Two patients had midnormal hepcidin levels. One was a young child who had normal urinary hepcidin measured on 2 occasions. The data on hepcidin levels in children are lacking, but we found similar levels in 2 healthy children. The affected child, however, suffers from frequent pharyngeal infections. The second patient with normal hepcidin levels was healing from multiple bone fractures and had still increased erythrocyte sedimentation rate. It is likely that the higher hepcidin levels in the 2 cases relative to other *TFR2* patients are related to chronic inflammatory conditions. This would suggest that *TFR2*-deficient subjects have low basal levels of hepcidin and inappropriate response to iron loading but can still respond to inflammation by increasing hepcidin production. However, even in these 2 cases, the hepcidin levels are lower than those observed in adults with inflammation.[12,15] In addition, the hepcidin-ferritin ratio in case 9 was significantly reduced as compared with healthy controls and was similar to the ratio in other *TFR2* patients. In agreement with our findings, down-regulation of hepcidin mRNA has been recently documented in *Tfr2*-deficient mice with the phenotype of hemochromatosis. In the same model, expression of hepcidin mRNA was induced by interleukin-6 (IL-6) and lipopolysaccharide (LPS).[16]

The ratio of urinary hepcidin to ferritin could be a useful index for assessing inadequate hepcidin responses to iron loading in hemochromatosis. The hepcidin-ferritin ratio is much less than 1 in nearly all of the patients with *TFR2* hemochromatosis (with the exception of patient 7) but is close to 1 in heterozygotes and controls. In iron disorders other than hemochromatosis, this ratio is also close to 1.[12] An alternative explanation for normal hepcidin levels observed in patient 7 might be that cumulative iron loading over many years is required to raise serum ferritin sufficiently for the hepcidin-ferritin ratio to become aberrant. At the age of 3 years, this may not have yet taken place (Table 1 and Figure 1).

The low or absent hepcidin levels in TFR2 patients resemble those observed in juvenile hemochromatosis. Like juvenile hemochromatosis patients, TFR2 patients have an early disease presentation,[12,17] but they run a clinical course less severe than that seen in juvenile hemochromatosis.[18]

In agreement with the observation of a direct correlation between hepcidin and *TFR2* mRNA expression in the liver,[19] we speculate that TFR2 contributes to the basal hepcidin production, likely in response to transferrin saturation. Recent findings indicate that TFR2 might be a sensor of transferrin saturation, because the TFR2 protein is stabilized in vitro in the presence of diferric transferrin.[20-21] This would be of particular significance for our findings, because all the TFR2 patients showed increased transferrin saturation. However, the presence of normal *TFR2* does not compensate for HFE dysfunction in iron-loaded patients with C282Y *HFE* mutations, indicating that hepcidin regulation by iron involves 2 parallel and partially redundant pathways. Conversely, in TFR2-deficient subjects, despite normal *HFE*, increased transferrin saturation did not induce hepcidin, except to a very limited extent in the 3 noncompliant patients with high serum ferritin.

Our results highlight that hepcidin is deficient in most genetic types of hemochromatosis. Thus, the lack of appropriate hepcidin response to iron loading could be a unifying diagnostic test for all these disorders.

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