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Original Article
Title:
Measurement of serum hepcidin-25 levels as a potential test for diagnosing hemochromatosis and related disorders

Short title:
Hepcidin in iron overload syndromes

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Abstract

Background. Iron overload syndromes include a wide spectrum of genetic and acquired conditions. Recent studies suggest suppressed hepcidin synthesis in the liver to be the molecular basis of hemochromatosis. However, a liver with acquired iron overload synthesizes an adequate amount of hepcidin. Thus, hepcidin could function as a biochemical marker for differential diagnosis of iron overload syndromes.

Methods. We measured serum iron parameters and hepcidin-25 levels followed by sequencing HFE, HJV, HAMP, TFR2, and SLC40A1 genes in 13 Japanese patients with iron overload syndromes. In addition, we performed direct measurement of serum hepcidin-25 levels using liquid chromatography–tandem mass spectrometry in 3 Japanese patients with aceruloplasminemia and 4 Italians with HFE-hemochromatosis.

Results. One patient with HJV-hemochromatosis, 2 with TFR2-hemochromatosis, and 3 with ferroportin disease were found among the 13 Japanese patients. The remaining 7 Japanese patients showed no evidence for genetic basis of iron overload syndrome. As far as the serum hepcidin-25 was concerned, seven patients with hemochromatosis and 3 with aceruloplasminemia showed markedly decreased serum hepcidin-25 levels. In contrast, 3 patients with ferroportin disease and 7 with secondary iron overload syndromes showed serum hepcidin levels parallel to their hyperferritinemia. Patients with iron overload syndromes were divided into 2 phenotypes presenting as low and high hepcidinemia. These were then associated with their genotypes.

Conclusion. Determining serum hepcidin-25 levels may aid differential diagnosis of iron overload syndromes prior to genetic analysis. (231 words)

Key Words: hepcidin, ceruloplasmin, hemochromatosis
Introduction

Hemochromatosis is caused by inadequate iron absorption leading to excessive body iron stores. If left undetected and untreated, progressive iron overload may independently lead to cirrhosis, diabetes mellitus (DM), cardiac failure, and endocrine disorders. Hemochromatosis includes HFE-hemochromatosis mainly from the C282Y/C282Y mutation in HFE.\(^1\) Non-HFE hemochromatosis is associated with the hemujuvelin (HJV),\(^2\) human antimicrobial peptide (HAMP),\(^3\) and transferrin receptor 2 (TFR2)\(^4\) genes. Evidence suggests the crucial role played by the hormone hepcidin, encoded by HAMP, in hemochromatosis. Hepcidin was first identified as an antimicrobial peptide synthesized in the liver and excreted in the urine.\(^5, 6\) Subsequent animal model studies indicated a close association of hepcidin with iron overload.\(^7, 8\) Hepcidin forms a complex with the transmembrane iron exporter protein of ferroportin; the complex is then internalized, and degraded in reticuloendothelial cells and enterocytes.\(^9\) Thus, hepcidin regulates body iron levels by functionally suppressing ferroportin. Pathological inflammation also increases hepcidin synthesis,\(^8, 10\) while erythropoietic activity decreases it.\(^11\) However, regardless of body iron overload, hepcidin mRNA expression in the liver is suppressed in patients with hemochromatosis,\(^12\) resulted in decreased urinary output of hepcidin.\(^2, 13, 14\) Evidence suggests that functional disruption of the hepcidin system might be a molecular basis for hemochromatosis, but few reports on the active form of serum hepcidin-25 have been published to date owing to the lack of reliable methods for quantitative determination.\(^15, 16, 17\)

Aceruloplasminemia is a neurological disorder characterized by heavy iron overload in the liver and brain with clinical manifestations of ataxia, involuntary movement, retinal degeneration, and dementia around the age of 50.\(^18\) It results from a mutation in the ceruloplasmin gene (CP).\(^19\) Ceruloplasmin encoded by CP is a major ferroxidase in circulation,
and is believed to play a crucial role in maintaining stability of cell surface ferroportin. Impaired regulation of ceruloplasmin negatively impacts ferroportin expression, which in turn blocks iron transport in patients.

Ferroportin disease has been recently identified as a genetic iron overload syndrome. The disease is inherited as an autosomal dominant trait, and occurs in populations throughout the world. Patients with a major form of ferroportin disease (type A) present with macrophage iron deposition and high ferritin levels despite normal transferrin saturation. In contrast, patients with the minor form (type B) develop abnormalities, such as elevated transferrin saturation and a more severe, mixed iron overload in parenchymal and reticuloendothelial cells similar to typical hemochromatosis. It is interesting that the urinary output of hepcidin increases in type A and is normal in type B ferroportin disease.

Transfusions and parenteral iron supplements are relatively frequent causes of secondary iron overload in patients with refractory anemia. Before the clinical introduction of erythropoietin, the anemia associated with chronic renal failure (CRF) was treated with transfusions or inadequate iron supplements leading to a variety of hyperferritinemias. Patients with chronic hepatitis C (CHC) may also face complications with parenchymal cell iron overload in the liver. Iron overload is frequently observed in patients with alcoholic liver disease (ALD) or nonalcoholic steatohepatitis (NASH). Thus, some patients with anemia, CRF, CHC, ALD, and NASH present with hyperferritinemia and liver iron deposition mimicking hemochromatosis and hence, require a differential diagnosis from genetic iron overload conditions. The liver with acquired iron overload can synthesize an appropriate amount of hepcidin. Therefore, direct measurement of serum hepcidin-25 levels could differentiate acquired iron overload from its genetic form, and also confirm results from current investigations, which are based on either
hepcidin mRNA expression in the liver or urinary output of hepcidin. Of the 3 types of hepcidin molecules, namely hepcidins-20, -22, and -25, the active form that binds to ferroportin is hepcidin-25. In this study, we evaluated serum hepcidin-25 levels as a biochemical marker to differentiate among iron overload disorders in 20 patients with established genetic basis of iron overload syndrome.
Methods

Twenty patients were enrolled in this study and were divided into 3 groups. The first group comprised of 13 Japanese patients who were referred to 2 institutes in central Japan (Kanazawa University Hospital and Aichi Gakuin University) between January 1998 and December 2009 for a genetic study of patients with iron overload syndromes. The second group comprised of 3 Japanese patients with aceruloplasminemia. Their condition had been effectively managed post diagnosis at Hamamatsu University Hospital. The clinical diagnosis of the 3 patients with aceruloplasminemia was confirmed by gene analyses for \( CP \) as per procedures approved by the ethics committees of Hamamatsu University (No. HUH 21-91) before the entry time of February of 2008 for direct determination of serum hepcidin-25. The third group comprised 4 of Italian patients who were randomly selected from an HFE hemochromatosis database at the University of Milano–Bicocca. All patients gave their informed written consent to genetic testing, measurement of all biochemical markers including serum hepcidine-25 levels and treatment according to the Declaration of Helsinki.

A biochemical study including determination of serum hepcidin-25 levels as well as a genetic analysis (prediagnostic fresh sampling) were performed in the first group of patients. The inclusion criterion was a biochemical iron overload twice as high as the normal range for serum ferritin levels (normal for male: 26–310 ng/mL; females: 7–110 ng/mL). The exclusion criterion was anemia less than 11.0 g/dL. Other biochemical tests included the measuring hemoglobin, serum iron levels, and iron-binding capacity. Transferrin saturation was estimated by the standard method: serum iron (\( \mu g/dL \))/iron-binding capacity (\( \mu g/dL \)) multiplied by 100.

The genes analyzed in the first group of patients included \( HFE, TFR2, HJV, HAMP, \) and \( SLC40A1 \). Informed consent was obtained from each patient, and the protocol was...
approved by the ethics committees of 2 institutes (Kanazawa University Hospital, No. 2004-66; and Aichi Gakuin University, Nos. 6 and 8). And the protocol was approved by the ethics committees of 2 institutes (Kanazawa University Hospital, No. 2004-66; and Aichi Gakuin University, Nos. 6 and 8).

In aceruloplasminemia patients, fresh sera for measuring serum hepcidin-25 levels were sampled between January and February 2008, during the post diagnosis management period. Iron overload persisted in these patients because of intolerance to phlebotomy treatment (fresh sampling during treatment). Long-term frozen sera obtained from the third group of Italian patients during the pre- and post-treatment periods were sent from Italy to an institute in Japan in February 2008 followed by measuring serum hepcidin-25 levels (long-term frozen sera).

Biochemical variables including serum hepcidin-25 levels were determined during phlebotomy treatment in 4 patients (cases 3, 5, 18, and 20).

Quantification of serum hepcidin-25 by LC tandem MS.

Sera from all patients were frozen and stored at −80 °C until analysis. Serum hepcidin-25 levels were determined using a liquid chromatography–tandem mass spectrometry-based assay system.35, 36 Rat serum spiked with human synthetic hepcidin-25 (MW2789, Peptide Inc., Minoh-shi, Japan) was used to obtain 1, 2, 4, 8, 16, 32, 64, and 128 ng/mL standards. Synthetic isotopic human hepcidin-25 (MW2830; Peptide Institute) was added to each diluted sample and to the standards as an internal standard. The samples and standards were then injected onto a 150 × 2.1 mm PLRP-S column packed with 5-mm particles with a 300-Å pore size (Varian Inc., CA, USA) for LC (Prominence LC20-ADvp; Shimadzu, Kyoto, Japan). The column eluent was connected to an ion spray source on a hybrid triple-quadrupole...
linear ion trap system (400 QTRAP LC/MS/MS System; Applied Biosystems, Foster City, CA, USA). A standard curve was generated to determine the mass spectrometer response for human synthetic hepcidin-25 under the described assay conditions. The curve was found to be linear from 1.0 to 128 ng/mL, \( y = 0.008x + 0.002, \ r = 0.998 \). Intraassay and interassay CVs were <6.7% and <8.8%, respectively. The lower limit of detection was 1.0 ng/mL with a signal to noise ratio of 10:1.

**Statistical analysis**

All pretreatment biochemical data were expressed as mean ± SD. Statistical analysis was performed using the nonparametric test (Spearman's rank method) to determine the strength of a correlation between serum ferritin and serum hepcidin-25 level.
Results

Patient profiles and laboratory data at entry are indicated in Table 1. The serum ferritin levels were $2680 \pm 2595$ ng/mL (range, 635–10,191 ng/mL), whereas transferrin saturation ranged from 11.2 to 99.0% as severity of aceruloplasminemia corresponded with the severity of iron deficiency. Five patients, including those with aceruloplasminemia, were mildly anemic despite the iron overload. One of the 15 patients who had undergone a liver biopsy had exceptional liver histology, which was almost normal in structure and showed minimal iron overload in the Kupffer cells (Case 11). Six patients (Cases 1–4, 6, 7) had advanced fibrosis or cirrhosis associated with parenchymal or mixed iron overload of hepatocytes and Kupffer cells. In contrast to heavy iron deposits, one patient (Case 13) had mild portal fibrosis and 3 patients (Cases 8-10) were free from fibrosis in the liver. In addition, 8 patients had DM, while only 1 patient had a triad of hemochromatosis of cirrhosis, DM, and pigmentation.

The results of the genetic study, serum hepcidin-25 levels, and final diagnoses of the iron overload syndromes are summarized in Table 2. The patients with iron overload syndromes were divided into 2 groups. Ten patients had serum hepcidin-25 levels below the upper limit of the normal range of 20 ng/mL (low group; Cases 1–10), while 10 patients had a level higher than 40 ng/mL (high group; Cases 11–20). A statistically significant difference between the low and high hepcidin groups was also observed ($7.4 \pm 5.8$ vs $138.6 \pm 62.6$ ng/mL; $P < 0.01$: Mann–Whitney test). This grouping was supported by another parameter of hepcidin/ferritin ratio that could be an iron regulatory hormone index adjusted by the representative body iron stores. The indices ranged between $0.05$ and $16.0 \times 10^{-3}$ in the low hepcidin group, and between $19.6$ and $148.8 \times 10^{-3}$ in the high hepcidin group. The low hepcidin group included 3 Japanese (Cases 1–3) and 4 Italian (Cases 4–7) patients with non-HFE and HFE hemochromatosis,
respectively. Three Japanese aceruloplasminemic patients (Cases 8–10) had low serum hepcidin-25 levels similar to those found in hemochromatosis.

The high hepcidin group included 2 families with ferroportin types A and B, respectively. The 49-year-old proband (Case 11) who exhibited selective iron overload in the reticuloendothelial cells, had a serum hepcidin-25 level of 42.5 ng/mL, while his 81-year-old father (Case 12) had a serum hepcidin-25 level of 155 ng/mL comparable with his high ferritin level of 2636 ng/mL. Another 66-year-old male patient (Case 13) of the second ferroportin disease family exhibited marked iron overload in the liver, which was first detected by CT imaging and later confirmed as heavy, mixed type-iron overload by biopsy. His serum hepcidin-25 level was 156.7 ng/mL and his serum ferritin level was also high (7980 ng/mL) with 89.2% transferrin saturation. The remaining 7 patients in the high hepcidin group had a variety of iron overload syndromes. One patient with CRF and DM (Case 14) and 3 patients with CHC (Cases 15–17) showed high serum hepcidin-25 levels corresponding to the biochemical iron markers. Mild iron overload associated with a high serum hepcidin-25 level was found in a patient with ALD (Case 19). Two patients (Cases 16 and 20) with a history of repeated use of iron supplements also had high serum hepcidin-25 levels. The \( HFE, HJV, HAMP, TFR2, \) and \( SLC40A1 \) analyses on these 7 patients excluded a genetic basis for their iron overloads; therefore, we concluded that they all had acquired iron overload liver diseases.

As shown in Figure 1, iron overload syndromes were clearly divided into two groups, but no correlations were observed between serum ferritin and hepcidin-25 levels in the high hepcidin group \( (p=0.096) \), nor low hepcidin group \( (p=0.349) \). Serum ferritin levels presented in a wide range \( (885–10,191 \text{ ng/mL}) \), while all serum hepcidin-25 levels were below \( 20 \text{ ng/mL} \) in the low hepcidin group. In contrast, both serum ferritin levels and hepcidin-25 levels in the high
hepcidin group presented in wide ranges of 635–7,980 ng/mL and 42.5–238.7 ng/mL, respectively.

Responses to serum hepcidin-25 to iron removal by phlebotomy are indicated in Figure 2. All patients responded similarly to the treatment, but differed in their serum hepcidin-25 levels. In 2 patients with TFR2 (Case 3) and HFE hemochromatosis (Case 5), pretreatment serum hepcidin-25 levels were low and declined slightly after treatment. In contrast, pretreatment serum hepcidin-25 levels were high and showed a marked reduction after treatment in the other 2 patients with secondary iron overload syndromes (Cases 18 and 20).
Discussion

This is the first report describing serum hepcidin-25 levels in patients with a variety of iron overload syndromes to the best of our knowledge. Because genetic iron overload syndromes showed an ethnic difference with regard to prevalence,\textsuperscript{19, 37, 38} the patient population was intentionally expanded to a wider range than the general population. Four Italian patients with HFE hemochromatosis and 3 Japanese patients with aceruloplasminemia were included in the study with 13 Japanese patients with a variety of iron overload syndromes referred to 2 institutes in central Japan. Patients with chronic inflammations such as CRF, CHC, and rheumatoid arthritis as well as patients with mild anemia who were on iron supplements were included. However, patients with severe anemia and acute phase inflammation were excluded from the study because hepcidin synthesis is decreased by erythropoietic activity and increased by acute inflammation.\textsuperscript{8, 11}

Although this study with its small sample size appears preliminary, the iron overload syndromes in patients could still be divided into 2 phenotypes, one with high and the other with low hepcidinemia, both closely linked to their respective genotype; this information may thus be crucial prior to performing genetic analysis. This study suggests that low hepcidin groups include individuals with hemochromatosis and aceruloplasminemia, while high hepcidin groups include those with ferroportin disease and secondary iron overload syndromes. Hemoglobin, serum ferritin levels, and iron saturation of transferrin have their limitations as grouping variables for iron overload syndromes. The direct measurement of serum hepcidin-25 levels are consistent with the suppressed expression of hepcidin mRNA in the livers of patients with HFE hemochromatosis\textsuperscript{12} and measurements of the urinary output of hepcidin in patients with HJV hemochromatosis\textsuperscript{2} as well as TFR2 hemochromatosis.\textsuperscript{14} HAMP hemochromatosis could also be
classified in this group on detecting decreased urinary output of hepcidin in such patients.\textsuperscript{13}

Inadequate serum hepcidin levels and a response to phlebotomy similar to that observed in this study were reported for HFE hemochromatosis recently.\textsuperscript{16}

This is the first study of its kind to demonstrate low serum hepcidin-25 levels in patients with aceruloplasminemia. Similar presentations resulting from body iron overload, such as low hepcidin synthesis in the liver and low transferrin saturation are reported in ceruloplasmin knockout mice.\textsuperscript{39} Ceruloplasmin is a multicopper oxidase and plays a role in the mobilization and oxidation of iron from tissue stores with subsequent incorporation of ferric iron into transferrin.\textsuperscript{18, 19, 20} According to the iron sensor hypothesis of TFR2, increasing concentrations of iron-saturated transferrin increase TFR2 protein levels in hepatocyte by protecting the receptor from degradation. And consecutively, TFR2 would be expected to increase the association with HFE and to stimulate of hepcidin transcription.\textsuperscript{40, 41} This hypothesis suggests a crucial role of low iron saturation of transferrin in falsely signaling iron deficiency to the liver in patients with aceruloplasminemia. This results in suppressed synthesis and secretion of hepcidin into blood. Further studies are needed to clarify the involvement of a low-set hepcidin system in increased iron absorption in the gut of patients with aceruloplasminemia.

Serum hepcidin appears to be regulated at a higher level in patients with acquired iron overload syndromes than in the hemochromatosis group. Serum hepcidin levels were also adequately controlled in our patients with ferroportin disease type A and B. Age-dependent iron overload in the type A proband and his father (Cases 11 and 12) was associated with greater hyperhepcidinemia. These blood test results were consistent with findings in previous reports that measured urinary output of hepcidin.\textsuperscript{27} Serum hepcidin was 156.7 ng/mL in another patient (Case 13) whose liver histology was compatible with type B ferroportin disease. In patients with
type A disease, SLC40A1 mutations generate a protein that is defective in cell surface expression or loss of iron export function.42, 43, 44 In the less prevalent type B, the mutant gene generates proteins that show normal cell surface expression but reduced sensitivity to hepcidin.42, 43 The resistance of ferroportin to hepcidin might be the key not only to the iron overload of ferroportin disease, but also to the secondary iron overload syndromes. In fact, only 2 of the 7 patients had a history of repeated use of iron supplements followed by adequate hepcidin regulation. The increased serum hepcidin-25 levels appeared to have suppressed iron absorption in the gut. In patients with chronic diseases of the kidney, liver, and joints, the current state of hyperhepcidinemia might be induced by an iron overload and chronic inflammation, but the pathophysiologic processes leading to body iron accumulation during the long-term disease condition remain to be elucidated. Ferroportin proteins in the secondary iron overload syndromes may also be resistant to hepcidin, permitting persistent iron absorption in the gut.

The involvement of the hepcidin system in iron regulation in CHC patients was unclear and thus much debated.15, 17, 45, 46 Three of our patients coinfected with HCV had a hepcidin-resistant iron overload syndrome without the evidence of a genetic basis for the latter. Establishing the serum hepcidin-25 levels35, 36, 47 might clarify complex issues related to ferroportin disease and the secondary iron overload syndromes.

The pregenetic differential analysis of patients with iron overload syndromes should include the evaluation of ferritin, ceruloplasmin, and hepcidin-25 as 3 potential biochemical markers (Figure. 3). The first test should ideally determine serum ferritin levels. Then, the serum hepcidin-25 levels provide a guideline for a genetic study. Serum ceruloplasmin should be determined in the low hepcidin group to rule out a rare genetic disease with aceruloplasminemia. Aceruloplasminemia itself might be diagnostic, and further studies on hypohepcidinemia might
indicate a lifelong low-iron diet in either symptomatic or presymptomatic patients because of their intolerance of iron reduction therapy.\textsuperscript{48} For patients without aceruloplasminemia, gene analysis for hemochromatosis is highly recommended to confirm their genetic background. The primary target gene in Caucasians is \textit{HFE}\textsuperscript{31,37} and the secondary genes include \textit{TFR2}, \textit{HJV}, and \textit{HAMP}. However, the primary target gene for non-Caucasians would be \textit{TFR2}\textsuperscript{33,49} followed by \textit{HJV}, \textit{HAMP}, and \textit{HFE} because HFE hemochromatosis is rarely present in other ethnic groups.\textsuperscript{37}

As noted in our patients, a high regulatory hepcidin set-point suggests a diagnosis of ferroportin disease. Before final diagnosis of a secondary iron overload syndrome, genetic analysis of \textit{SLC40A1} is required. Without such a genetic study it is difficult to rule out the possible complication of this rare genetic disease with the more common, acquired iron overload syndromes.

In conclusion, measurement of the serum hepcidin-25 levels has the potential for grouping patients with iron overload syndromes based on a newly proposed iron regulatory system. When combined with ferritin and ceruloplasmin levels, hepcidin-25 levels could be a potential test for the diagnosis of hemochromatosis and related disorders.
References


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35 Murao N, Ishigai M, Yasuno H, Shimonaka Y, Aso Y. Simple and sensitive quantification


Table 1. The Clinical Features and Laboratory Data of Patients with Iron Overload Syndromes at Entry

<table>
<thead>
<tr>
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<th>Age/Sex</th>
<th>Clinical Diagnosis</th>
<th>Blood Tests</th>
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<td>Transferrin Saturation (%)</td>
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<tr>
<td>20</td>
<td>33/F</td>
<td>ISA</td>
<td>2076</td>
<td>91.1</td>
<td>12.6</td>
<td>normal/Pno</td>
</tr>
</tbody>
</table>

Subtotal from Pt.1 to 10 (Mean±SD) 2986±2978 68.1±38.4 13.3±2.8
Subtotal from Pt.11 to 20 (Mean±SD) 2375±2266 76.9±23.0 13.4±1.4
Total (Mean±SD) 2680±2595 72.5±31.2 13.3±2.1
Genetic study of the patient was reported in detail.\textsuperscript{1,30}
Genetic study of the patient was reported in detail.\textsuperscript{2,29}
Italian patient with HFE-hemochromatosis specially enrolled
Japanese patient with aceruloplasminemia specially enrolled
Genetic study of the patient was reported in detail.\textsuperscript{18}

Normal value of serum ferritin; male:26-310 ng/mL, female:7-110 ng/mL
Normal value of transferrin saturation; 40-70%
Normal value of hemoglobin; male:13.5-17.0 g/dL, female:11.2-14.5 ng/mL

DM; diabetes mellitus
M; male, F; female
P; parenchymal, RE; reticuloendothelial, mixed; parenchymal and reticuloendothelial
CRF; chronic renal failure
ISA; iron supplement for anemia of unknown etiology
CHC; chronic hepatitis C
CR; complete responder
IFN; interferon
RA; rheumatoid arthritis
Table 2. Results of Genetic Study, Serum Hepcidin Determination, and Final Diagnosis for Iron Overload Syndromes

<table>
<thead>
<tr>
<th>Pt.</th>
<th>Genetic Study</th>
<th>Serum Hepcidin-25 (ng/mL)</th>
<th>Hepcidin-25/Ferritin ($\times 10^{-3}$)</th>
<th>Final Diagnosis for Iron Overload Syndrome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>745G&gt;C/745G&gt;C in HJV*1</td>
<td>0.3</td>
<td>0.05</td>
<td>HJV-hemochromatosis</td>
</tr>
<tr>
<td>2</td>
<td>1469T&gt;G/1469T&gt;G in TFR2*2</td>
<td>2.8</td>
<td>2.7</td>
<td>TFR2-hemochromatosis</td>
</tr>
<tr>
<td>3</td>
<td>1100T&gt;G/2008-9delAC in TFR2*3</td>
<td>12.2</td>
<td>1.2</td>
<td>TFR2-hemochromatosis</td>
</tr>
<tr>
<td>4</td>
<td>845G&gt;A/845G&gt;A in HFE*4</td>
<td>3.0</td>
<td>1.4</td>
<td>HFE-hemochromatosis</td>
</tr>
<tr>
<td>5</td>
<td>845G&gt;A/845G&gt;A in HFE</td>
<td>6.7</td>
<td>3.6</td>
<td>HFE-hemochromatosis</td>
</tr>
<tr>
<td>6</td>
<td>845G&gt;A/845G&gt;A in HFE</td>
<td>4.1</td>
<td>1.9</td>
<td>HFE-hemochromatosis</td>
</tr>
<tr>
<td>7</td>
<td>845G&gt;A/845G&gt;A in HFE</td>
<td>3.1</td>
<td>1.0</td>
<td>HFE-hemochromatosis</td>
</tr>
<tr>
<td>8</td>
<td>607Ains/607Ains in Cp*5</td>
<td>10.3</td>
<td>11.6</td>
<td>aceruloplasminemia</td>
</tr>
<tr>
<td>9</td>
<td>2630G&gt;A/2630G&gt;A in Cp*5</td>
<td>13.1</td>
<td>12.7</td>
<td>aceruloplasminemia</td>
</tr>
<tr>
<td>10</td>
<td>1287TACACins/1287TACACins in Cp</td>
<td>18.2</td>
<td>16.0</td>
<td>aceruloplasminemia</td>
</tr>
<tr>
<td>11</td>
<td>1467A&gt;C/wt in SLC40A1*6</td>
<td>42.5</td>
<td>61.1</td>
<td>ferroportin disease type A</td>
</tr>
<tr>
<td>12</td>
<td>1467A&gt;C/wt in SLC40A1</td>
<td>155.0</td>
<td>58.8</td>
<td>ferroportin disease type A</td>
</tr>
<tr>
<td>13</td>
<td>470A&gt;C/wt in SLC40A1*7</td>
<td>156.7</td>
<td>19.6</td>
<td>ferroportin disease type B</td>
</tr>
<tr>
<td>14</td>
<td>no mutations responsible</td>
<td>181.0</td>
<td>116.2</td>
<td>$2^{nd}$ iron overload in CRF and DM</td>
</tr>
<tr>
<td>15</td>
<td>no mutations responsible</td>
<td>63.0</td>
<td>99.2</td>
<td>$2^{nd}$ iron overload in CHC</td>
</tr>
<tr>
<td>16</td>
<td>no mutations responsible</td>
<td>189.0</td>
<td>148.8</td>
<td>$2^{nd}$ iron overload in CHC with iron supplement</td>
</tr>
<tr>
<td>17</td>
<td>no mutations responsible</td>
<td>82.5</td>
<td>47.9</td>
<td>$2^{nd}$ iron overload in CHC</td>
</tr>
<tr>
<td>18</td>
<td>no mutations responsible</td>
<td>238.7</td>
<td>54.5</td>
<td>$2^{nd}$ iron overload in RA</td>
</tr>
<tr>
<td>19</td>
<td>no mutations responsible</td>
<td>105.5</td>
<td>132.0</td>
<td>$2^{nd}$ iron overload in alcoholic cirrhosis</td>
</tr>
<tr>
<td>20</td>
<td>no mutations responsible</td>
<td>172.5</td>
<td>83.1</td>
<td>$2^{nd}$ iron overload with iron supplement</td>
</tr>
</tbody>
</table>

Subtotal from Pt.1 to 10  (Mean±SD)  7.4±5.8  5.2±5.9  
Subtotal from Pt.11 to 20  (Mean±SD)  138.6±62.6  82.1±41.1  
Total  (Mean±SD)  73.0±80.0  43.7±48.7
*1 The 745 G>C in HJV predicts D249H in HJV protein.
*2 The 1469T>G in TFR2 predicts L490R in TFR2 protein.
*3 The 1100T>G in TFR2 predicts L367R in TFR2 protein.
*4 The 845G>A in HFE predicts C282Y in HFE protein.
*5 The 2630G>A in Cp predicts W858ter in Cp protein.
*6 The 1467A>C in SLC40A1 predicts R489S in ferroportin.
*7 The 470A>C in SLC40A1 predicts D175A in ferroportin.

CRF; chronic renal failure
CHC; chronic hepatitis C
DM; diabetes mellitus
RA; rheumatoid arthritis
**Figure 1.** Relationships between serum hepcidin-25 and ferritin levels of patients at entry.

Patients with ferroportin disease and secondary iron overload (indicated by closed circles) showed high hepcidin-25 levels, whereas patients with hemochromatosis and aceruloplasminemia (indicated by open circles) demonstrated low serum hepcidin-25, irrespective of high serum ferritin levels. The figure indicates that patients with iron overload syndromes are clearly divisible into high and low hepcidin groups. However, any correlations are not observed between serum hepcidin-25 level ($y$) and ferritin level ($x$) in the high hepcidin group ($y = 0.013x + 108, r = 0.46$), nor in the low hepcidin group.

**Figure 2.** Reduction of serum hepcidin-25 levels after phlebotomy.

Two patients each in the low hepcidin and high hepcidin groups responded similarly to phlebotomy treatment, but differ in serum hepcidin-25 levels. Serum hepcidin-25 levels are low in patients with hemochromatosis (Cases 3, 5) but high in patients with secondary iron overload syndromes (Cases 18, 20). Serum ferritin level in indicated in parentheses as ng/mL.
Serum hepcidin levels provide a guideline for the genetic study of iron overload syndromes. The first step in differential diagnosis might be to divide the patients into 2 groups with low and high serum hepcidin-25 levels, respectively. The low hepcidin group includes patients with hemochromatosis and a rare aceruloplasminemia, while the high hepcidin group consists of patients with ferroportin disease and acquired iron overload syndromes. Determination of serum ceruloplasmin might be needed before a genetic study of hemochromatosis. Aceruloplasminemia may be suspected from specific clinical features and direct determination of serum ceruloplasmin before hepcidin measurement. In patients with hyperhepcidinemia, genetic analysis for \textit{SLC40A1} is needed before the final diagnosis for secondary iron overload syndromes. The primary target gene in Caucasian hemochromatosis is \textit{HFE}, whereas in non-Caucasians it is \textit{TFR2}.
Iron Overload Syndrome (Two Times Normal Serum Ferritin)
(measure serum hepcidin 25)

High Hepcidin

Low Hepcidin

(measure serum ceruloplasmin)

Non-Aceruloplasminemia

Non Caucasian

Gene Analysis SLC40A1

2nd Iron Overload Syndrome

Ferroportin Disease

TFR2, HJV, HAMP, HFE, etc.

TFR2-Hemochromatosis, etc.

Caucasian

HFE, TFR2, HJV, HAMP, etc.

HFE-Hemochromatosis, etc.

Aceruloplasminemia

CP

Aceruloplasminemia

Non-Aceruloplasminemia

Aceruloplasminemia