Two novel mutations, L490R and V561X, of transferrin receptor 2 gene in Japanese patients with hemochromatosis

Chizu Koyama
Shinya Wakusawa
Hisao Hayashi
Rie Suzuki
Motoyoshi Yano
Kentaro Yoshioka
Mitsuo Kozuru
Yoshihiro Takayama
Toshhide Okada
Hiroshi Mabuchi

Background and Objectives. The low prevalence of the C282Y mutation of the HFE gene in Japan means that the genetic background of hemochromatosis in Japanese patients remains unclear. In a previous report, we showed that 3 patients from one family had an AVAQ 594-597 deletion of the transferrin receptor (TFR2) gene. This suggests that the TFR2 gene is involved in hemochromatosis in Japanese patients.

Design and Methods. Nine patients clinically diagnosed with hemochromatosis were included in the study. DNA was extracted from whole blood samples collected with informed consent. The HFE and TFR2 genes were analyzed by sequencing the coding region and splicing sites.

Results. There were no mutations in the HFE gene. In the TFR2 gene, 2 novel mutations, 1469T→G (L490R) and 1665delC (V561X), were found in 2 patients. A known variation, 714C→G (I238M), was also found in the patient with L490R. The patient homozygous for both L490R and I238M presented with a mild manifestation of hemochromatosis at the age of 41 years. His liver was cirrhotic with parenchymal iron deposits and the result of a glucose tolerance test was compatible with diabetes mellitus. The patient homozygous for V561X had severe iron overload with the triad of cirrhosis, diabetes mellitus and skin pigmentation at the age of 58 years.

Interpretations and Conclusions. Taken together with the previous report, 5 of our 12 patients with hemochromatosis manifesting in middle age had mutations in the TFR2 gene. Thus, TFR2 plays a role in the pathogenesis of hemochromatosis in Japan.

Key words: cirrhosis, diabetes, iron, liver, non-HFE.

Haematologica 2005; 90:302-306
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Iron is an essential element but an overload has toxic effects on the liver, pancreas and other organs. Hemochromatosis of unknown etiology has been postulated as a genetic defect in regulatory iron absorption. The recent identification of the key proteins involved in iron metabolism, namely HFE,1 DMT1,3 transferrin receptor 2 (TFR2),4 ferroportin 1,5 hepcidin1 and hemojuvelin,6 has provided new insight into hemochromatosis. The discovery of a single amino acid substitution, C282Y, in the HFE gene not only opened the door to investigations of regulatory iron absorption in the gut, but also introduced a genetic diagnosis for major iron overload disorders. Though its prevalence is high in Caucasians,6 hemochromatosis is a rare disorder in Orientals including Japanese. This may be accounted for by the low prevalence of the C282Y mutation of the HFE gene in Asians.7,8 Non-HFE hemochromatosis has been classified into subtypes based on genetic background.9 These include juvenile hemochromatosis (HFE2) of hepcidin10 and hemojuvelin,11 and middle-age-onset hemochromatosis with mutant genes for TFR2 (HFE3)12 and ferroportin 1 (HFE4).13 The first paper on a mutation of the hepatic TFR2 gene was published in Italy in 2000.14 Subsequent case reports from different ethnic groups suggested that TFR2 is responsible for one of the subtypes of middle-age-onset hemochromatosis.15-17 In a previous study, we reported an AVAQ 594-597 deletion of TFR2 in three siblings of one hemochromatosis family.18 This suggests that the TFR2 gene is involved in hemochromatosis in Japan. To examine this possibility further, we conducted mutation analysis of the HFE and TFR2 genes in 9 patients with hemochromatosis.

Design and Methods

A total of 9 patients with hemochromatosis of unknown etiology were enrolled in the current study. Heavy drinkers, subjects with viral hepatitis and
those who had received repeated transfusions were excluded. DNA was extracted from the peripheral blood cells of each patient after written informed consent had been obtained. For the analysis of mutations of the \textit{HFE} and \textit{TFR2} genes, the coding region and splicing sites were amplified by polymerase chain reaction (PCR) using the primers listed in Table 1, and the PCR products were sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit and ABI PRISM 3100/Avant Genetic Analyzer (Applied Biosystems). When a novel mutation was found, the mutation was screened for in 50 healthy volunteers. In one case in which the patient was homozygous for a novel mutation, family members were also subjected to investigation. This study was conducted in accordance with the ethical guidelines for human genome/gene analysis research by the Ministry of Education, Culture, Sports, Science and Technology, the Ministry of Health, Labor and Welfare, and the Ministry of Economy, Trade and Industry, Japan.

\begin{table}
\centering
\caption{Primer sets for PCR and sequencing.}
\begin{tabular}{|c|c|c|}
\hline
& \textbf{Forward primer} & \textbf{Reverse primer} \\
\hline
\textbf{HFE} & & \\
Exon 1 & 5'-AGA TCA GAC CAG CCT TGG TTC AG -3' & 5'-TTT GGA CCA CCG GGC CG -3' \\
Exon 2 & 5'-ATG GGC CAC TGA TGG CA -3' & 5'-GAA GAG CTC TGA CCA CTT GA -3' \\
Exon 3 & 5'-CTC CAG AGA AGT GAA GCA GGC CT -3' & 5'-CCA CTC CAG AGA GCT AAT CT -3' \\
Exon 4 & 5'-CTG CCT TGC TCT TCC CTT CC -3' & 5'-TTG CGA CCA CCG GCG CG -3' \\
Exon 5 & 5'-CTG CCT TGC TCT TCC CTT CC -3' & 5'-TTG CGA CCA CCG GCG CG -3' \\
Exon 6 & 5'-CTG CCT TGC TCT TCC CTT CC -3' & 5'-TTG CGA CCA CCG GCG CG -3' \\
Exon 7 & 5'-CTG CCT TGC TCT TCC CTT CC -3' & 5'-TTG CGA CCA CCG GCG CG -3' \\
Exon 8 & 5'-CTG CCT TGC TCT TCC CTT CC -3' & 5'-TTG CGA CCA CCG GCG CG -3' \\
Exon 9 & 5'-CTG CCT TGC TCT TCC CTT CC -3' & 5'-TTG CGA CCA CCG GCG CG -3' \\
Exon 10 & 5'-CTG CCT TGC TCT TCC CTT CC -3' & 5'-TTG CGA CCA CCG GCG CG -3' \\
Exon 11 & 5'-CTG CCT TGC TCT TCC CTT CC -3' & 5'-TTG CGA CCA CCG GCG CG -3' \\
Exon 12 & 5'-CTG CCT TGC TCT TCC CTT CC -3' & 5'-TTG CGA CCA CCG GCG CG -3' \\
Exon 13 & 5'-CTG CCT TGC TCT TCC CTT CC -3' & 5'-TTG CGA CCA CCG GCG CG -3' \\
Exon 14 & 5'-CTG CCT TGC TCT TCC CTT CC -3' & 5'-TTG CGA CCA CCG GCG CG -3' \\
Exon 15 & 5'-CTG CCT TGC TCT TCC CTT CC -3' & 5'-TTG CGA CCA CCG GCG CG -3' \\
Exon 16 & 5'-CTG CCT TGC TCT TCC CTT CC -3' & 5'-TTG CGA CCA CCG GCG CG -3' \\
Exon 17 & 5'-CTG CCT TGC TCT TCC CTT CC -3' & 5'-TTG CGA CCA CCG GCG CG -3' \\
Exon 18 & 5'-CTG CCT TGC TCT TCC CTT CC -3' & 5'-TTG CGA CCA CCG GCG CG -3' \\
\hline
\textbf{TFR2} & & \\
Exon 1 & 5'-GGA GAG GAC CAG CCT TGG TTC AG -3' & 5'-TTT GGA CCA CCG GGC CG -3' \\
Exon 2 & 5'-ATG GGC CAC TGA TGG CA -3' & 5'-GAA GAG CTC TGA CCA CTT GA -3' \\
Exon 3 & 5'-CTC CAC AGA AGG GAA GCA GGC CT -3' & 5'-CCA CTC CAG AGA GCT AAT CT -3' \\
Exon 4 & 5'-AGG TCT TGT GGA CCA CCG GGC CG -3' & 5'-GAA GAG CTC TGA CCA CTT GA -3' \\
Exons 5,6 & 5'-GCC ATC AGC GCA GAG GGT GGC GT -3' & 5'-GAA GAG CTC TGA CCA CTT GA -3' \\
Exons 7,8 & 5'-GCC ATC AGC GCA GAG GGT GGC GT -3' & 5'-GAA GAG CTC TGA CCA CTT GA -3' \\
Exons 9,10 & 5'-GCC ATC AGC GCA GAG GGT GGC GT -3' & 5'-GAA GAG CTC TGA CCA CTT GA -3' \\
Exons 11-13 & 5'-GCC ATC AGC GCA GAG GGT GGC GT -3' & 5'-GAA GAG CTC TGA CCA CTT GA -3' \\
Exons 14,15 & 5'-GCC ATC AGC GCA GAG GGT GGC GT -3' & 5'-GAA GAG CTC TGA CCA CTT GA -3' \\
Exons 16 & 5'-GCC ATC AGC GCA GAG GGT GGC GT -3' & 5'-GAA GAG CTC TGA CCA CTT GA -3' \\
Exons 17 & 5'-GCC ATC AGC GCA GAG GGT GGC GT -3' & 5'-GAA GAG CTC TGA CCA CTT GA -3' \\
Exons 18 & 5'-GCC ATC AGC GCA GAG GGT GGC GT -3' & 5'-GAA GAG CTC TGA CCA CTT GA -3' \\
\hline
\end{tabular}
\end{table}

\begin{figure}
\centering
\includegraphics[width=0.8\textwidth]{figure1}
\caption{(left). Sequence traces in the region of 1469T$\rightarrow$G (L490R) in patient 7. The figure shows a normal control and a homozygote for 1469T$\rightarrow$G identified in exon 11. The mutation substitutes a leucine with arginine at position 490 of the receptor protein (L490R).}
\end{figure}
The clinical features of the 9 unrelated patients with hemochromatosis and the results of the analysis of the TFR2 gene are summarized in Table 2. One patient had a brother who died of hemochromatosis before our analysis. The remaining 8 patients had no relatives suffering from hemochromatosis. None of our patients had any mutation of the HFE gene responsible for iron overload, including C282Y. Two of the 9 patients had novel TFR2 mutations, 1469T→G and 1665delC. Patient #7, of a non-consanguineous family, was homozygous for 1469T→G in exon 11. As shown in Figure 1, 1469T→G resulted in a leucine to arginine substitution at position 490 in the protein.

**Table 2. Clinical features and mutations of patients.**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yrs)</th>
<th>Sex</th>
<th>Cirrhosis</th>
<th>DM</th>
<th>Pigmentation</th>
<th>Ferritin (ng/mL)</th>
<th>Transferrin saturation (%)</th>
<th>Mutations of the TFR2 gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>48</td>
<td>M</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>6115</td>
<td>94.8</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>51</td>
<td>F</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>4278</td>
<td>95.8</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>45</td>
<td>M</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>3000</td>
<td>94.4</td>
<td>714C→G / wild</td>
</tr>
<tr>
<td>4</td>
<td>58</td>
<td>M</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>1982</td>
<td>88.7</td>
<td>1665delC / 1665delC*</td>
</tr>
<tr>
<td>5</td>
<td>63</td>
<td>M</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
<td>1443</td>
<td>90.1</td>
<td>–</td>
</tr>
<tr>
<td>6</td>
<td>47</td>
<td>M</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
<td>3489</td>
<td>94.2</td>
<td>–</td>
</tr>
<tr>
<td>7</td>
<td>41</td>
<td>M</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
<td>2040</td>
<td>93.6</td>
<td>714C→G / wild</td>
</tr>
<tr>
<td>8</td>
<td>45</td>
<td>F</td>
<td>no</td>
<td>no</td>
<td>yes</td>
<td>4485</td>
<td>52.9</td>
<td>–</td>
</tr>
<tr>
<td>9</td>
<td>55</td>
<td>M</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>2735</td>
<td>74.4</td>
<td>–</td>
</tr>
</tbody>
</table>

DM; diabetes mellitus; *novel mutation responsible for hemochromatosis.

**Figure 2.** Sequence traces in the region of 1665delC (V561X) in patient 4. The figure shows a normal control and a homozygote for 1665delC identified in exon 14. The mutation caused a frame-shift change creating a premature stop codon at the 561st amino acid valine (V561X).
Mutations in transferrin receptor 2 gene

A table listing variations of the TFR2 gene reported in non-HFE hemochromatosis.

Table 3. Variations of the TFR2 gene reported in non-HFE hemochromatosis.

<table>
<thead>
<tr>
<th>Variation</th>
<th>Amino acid</th>
<th>Type</th>
<th>Exon</th>
<th>Region</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutations</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>84-88insC</td>
<td>E60X</td>
<td>Frameshift</td>
<td>2</td>
<td>Southern Italy</td>
<td>Roetto et al. 14</td>
</tr>
<tr>
<td>515T→A</td>
<td>M172K</td>
<td>Missense</td>
<td>4</td>
<td>Southern Italy</td>
<td>Roetto et al. 14</td>
</tr>
<tr>
<td>750C→G</td>
<td>Y250X</td>
<td>Nonsense</td>
<td>6</td>
<td>Sicily</td>
<td>Camaschella et al. 13</td>
</tr>
<tr>
<td>1780-91del</td>
<td>AWAQ594-7del</td>
<td>Deletion</td>
<td>16</td>
<td>Italy</td>
<td>Roetto et al. 14</td>
</tr>
<tr>
<td>2069A→C</td>
<td>Q69OP</td>
<td>Missense</td>
<td>17</td>
<td>Portugal</td>
<td>Piperno et al. 21</td>
</tr>
<tr>
<td>1469T→G</td>
<td>L490R</td>
<td>Deletion</td>
<td>14</td>
<td>Japan</td>
<td>Gielli et al. 16</td>
</tr>
<tr>
<td>1665delC</td>
<td>V561X</td>
<td>Missense</td>
<td>11</td>
<td>Sicily</td>
<td>Hattori et al. 18</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probable polymorphisms</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1391G→A</td>
<td>R455Q</td>
<td>Missense</td>
<td>10</td>
<td>Asia</td>
<td>Hofmann et al. 22</td>
</tr>
<tr>
<td>64G→T</td>
<td>V22I</td>
<td>Missense</td>
<td>2</td>
<td>China</td>
<td>Biasiottto et al. 23</td>
</tr>
<tr>
<td>714C→G</td>
<td>I238M</td>
<td>Missense</td>
<td>5</td>
<td>Japan</td>
<td>Lee et al. 24</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The patient with this mutation had cirrhotic liver histology and diabetes, but was free from skin pigmentation at the age of 41 years. Histochemical iron deposited predominantly in the hepatocytes (18,725 µg/g wet weight). Patient #4, of a consanguineous family, was homozygous for 1665delC in exon 14. The mutation delC in codon 555 caused a frame-shift and created a premature stop codon at the 561st amino acid valine (V561X) as shown in Figure 2. The patient with V561X manifested the triad of cirrhosis, diabetes mellitus and skin pigmentation at the age of 58 years. In addition to the triad, he suffered from congestive heart failure when diagnosed. The 2 mutations L490R and V561X were not found in 50 healthy volunteers studied. In this study we also found the mutation 714C→G, which causes an amino acid substitution of isoleucine for methionine (I238M). The patient with L490R was homozygous for the known variation (Table 3) and his aged parents without iron overload were compound heterozygotes for L490R and I238M (Figure 3).

Discussion

Even though hemochromatosis is rare in Japan, 3 genotypes have been identified in Japanese patients. Homozygosity of the C282Y mutation in the HFE gene, a major mutant for Caucasians, was found in a hemochromatosis patient who was resident in Kyushu, a southwestern island of Japan. One family of 4 affected members with a novel mutation of the H chain of ferritin was reported from the northeast island of Hokkaido. In a previous study, we reported an AVAQ 594-597 deletion of TFR2 in a hemochromatosis family of the main island. As listed in Table 3, 5 mutations of TFR2 responsible for hemochromatosis have been reported in various ethnic groups including Caucasians, African Americans and Japanese. The 2 mutations L490R and V561X in TFR2 were found for the first time in our patients. The gene products altered by these novel mutations might induce TFR2 dysfunction, playing an important role in the pathogenesis of hemochromatosis. Lee et
reported I238M as a polymorphism because a woman homozygous for the mutation had no signs of iron overload. Our family study on the aged parents with the compound heterozygous mutations I238M and L490R supported the hypothesis that I238M is a polymorphism provided that L490R is responsible for the iron overload of recessive inheritance. The 3 siblings with AVAQ 594-597 deletion reported previously showed a mild iron overload in their 50s. The eldest male sibling was heterozygous for the mutation but free from an iron overload disorder. The patient with L490R manifested 2 features of the triad at the age of 41 years. His aged parents were free from iron overload. The patient with V561X manifested the hemochromatosis triad at the age of 58 years. He was a member of a consanguineous family, but a genetic study was not conducted for ethical reasons. Thus, as far as we patients with TFR2 gene mutations are concerned, the third type of hemochromatosis (HFE3) is characterized by a male dominant, middle-aged onset iron overload with autosomal recessive inheritance. Taken together with 3 patients in a previous report, 5 of our 12 patients were homozygous for the responsible mutations in the TFR2 gene. Because of the relatively high prevalence of the mutant gene for the hepatic transferrin receptor, this might play an important role in Japanese patients with hemochromatosis. Further studies are required to estimate the prevalence of TFR2 mutations in the general population and their effect on patients with chronic liver diseases other than hemochromatosis.

All the authors contributed to the design of the study and to the conception of the experimental work. All authors reviewed the manuscript and approved the final version to be submitted. A special thank to Dr H. Kawabata for critical review of this manuscript. The authors declare that they have no potential conflict of interest.

This study was supported by The Specific Research Fund of Hokuriku University. Manuscript received May 14, 2004. Accepted January 22, 2005.

References