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

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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.

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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,[2] transferrin receptor 2 (TfR2),[3] ferroportin 1,[4] hepcidin[5] 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,[7,8] 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.[7,9,10] Non-HFE hemochromatosis has been classified into subtypes based on genetic background.[11] These include juvenile hemochromatosis (HFE2) of hepcidin[12] and hemojuvelin,[6] and middle-age-onset hemochromatosis with mutant genes for TfR2 (HFE3[13] and ferroportin 1 (HFE4).[4] The first paper on a mutation of the hepatic *TFR2* gene was published in Italy in 2000.[13] Subsequent case reports from different ethnic groups suggested that *TFR2* is responsible for one of the subtypes of middle-age-onset hemochromatosis.[14-17] 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

Table 1. Primer sets for PCR and sequencing.

	Forward primer	*Reverse primer*
HFE		
Exon 1	5'- AGA TCA GAA CAT TGC GAA GC -3'	5'- TTT CGA TTT TTC CAC CCC CG -3'
Exon 2	5'- ATA GGG ACA TGG TTA AGG CC -3'	5'- GAA AAG CTC TGA CAA CCT CA -3'
Exon 3	5'- CCT ATT CCT TTG GTT GCA GT -3'	5'- CCA CTC TGC CAC TAG AGT AT -3'
Exon 4	5'- CAG ATC CCT CTC CTC ATC CT -3'	5'- TTT TCT CAG CTC CTG GCT CT -3'
Exon 5	5'- CTG CTC TTT GTT AGG GGA TG -3'	5'- GGC AGA GGT ACT AAG AGA CT -3'
Exon 6-1	5'- AAG ATG GTG CCT AGG TTT GT -3'	5'- TCC CCC AAA TTT AAG GAG TC -3'
Exon 6-2	5'- CCG TCA CCT CAG AGA CAT AC-3'	5'- AAA CAC CTC TTC ACA ACC CC -3'
Exon 6-3	5'- AGC ATC ATG GCT ATC TGT GG -3'	5'- GGA TGG TCT CGA TCT CCT GA -3'
TFR2		
Exon 1	5'- GAG GAG CAG CCT TGG TTC AG -3'	5'- AGA AGC GAG GTC AGG ACA CG -3'
Exon 2	5'- TCA CTG ACC TCA TTA TTG CC -3'	5'- AAG GCT GGC GGG TGG CAA GA -3'
Exon 3	5'- CTC CCC AGA AGT GAA GGG CT -3'	5'- GCA GAT GGG AGG ACT CAG GA -3'
Exon 4	5'- ACG TCT CTG GCA TCT TCC CT -3'	5'- GGT GAG CGC CCC GAG CCG CG -3'
Exons 4,5	5'- TTC CTA AAC TCA GGA ACC CC -3'	5'- TTC GAG ACC CAG GAA AGG -3'
Exons 5,6	5'- CTA CGT GGG GCT GCA ATT CC -3'	5'- TTC TCA CTG GCA GTC CGA CC -3'
Exon 6	5'- GAG CCC TTT CCT GGG TCT CGA A -3'	5'- CCT GAA CGA TTC TCA CTG GC -3'
Exons 7,8	5'- GTG GGA TGG ACA GTT GCA AG -3'	5'- GTT CAC CCA CAA TCA CCC TG -3'
Exon 9	5'- GCG ATC TGG AGC CAG AAA GG -3'	5'- TGC ACC AGC CCC CTA TCT TG -3'
Exon 10	5'- GAC ACA GGC AGA TGG AGG AT -3'	5'- TGT CCC TCA CTG CCT CTC TG -3'
Exon 11-13	5'- GGG GAC CAG GAC AGA AGA AG -3'	5'- GCA AGA GCA CCC CAG GAA TA -3'
Exons 14,15	5'- GCA AGA GCA CCC CAG GAA TA -3'	5'- GCT GAA GGA CTA GCG CTG TG -3'
Exon 16	5'- CCC AGC GTC CAC CCT GTC CTG GC -3'	5'- CTG GAT TGC CAG AGA GGA CC -3'
Exon 17	5'- GTC CTT GTC CCC ATG CTA GG -3'	5'- CAG AGG ACC GGG ACT GTG TA -3'
Exon 18	5'- AAA GAC TGG CTG GCG GGA AG -3'	5'- AGG GGA AGG GGC TGT GAT TG -3'

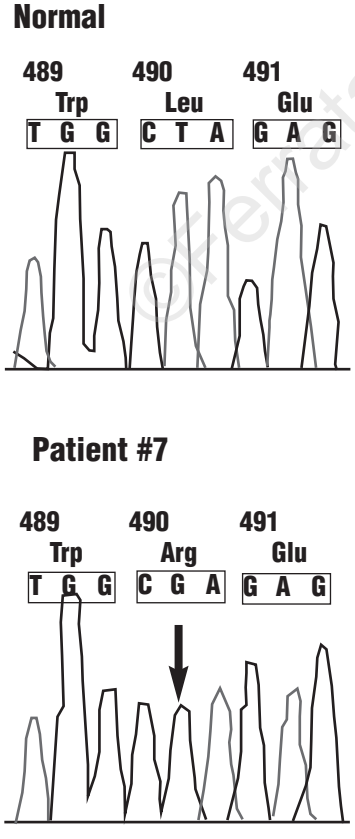

Figure 1 (left). Sequence traces in the region of 1469T→G (*L490R*) in patient 7. The figure shows a normal control and a homozygote for 1469T→G identified in exon 11. The mutation substitutes a leucine with arginine at position 490 of the receptor protein (L490R).

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 *HFE* and *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.

Table 2. Clinical features and mutations of patients.

Patient	Age (yrs)	Sex	Clinical manifestation			Ferritin (ng/mL)	Transferrin saturation (%)	Mutations of the TFR2 gene
			Cirrhosis	DM	Pigmentation			
1	48	M	yes	yes	yes	6115	94.8	–
2	51	F	yes	yes	yes	4278	95.8	–
3	45	M	yes	yes	yes	3000	94.4	714C→G / wild
4	58	M	yes	yes	yes	1982	88.7	1665delC / 1665delC*
5	63	M	no	yes	yes	1443	90.1	–
6	47	M	no	yes	yes	3489	94.2	–
7	41	M	yes	yes	no	2040	93.6	714C→G/714C→G, 1469T→G /1469T→G*
8	45	F	no	no	yes	4485	52.9	–
9	55	M	no	no	no	2735	74.4	–

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

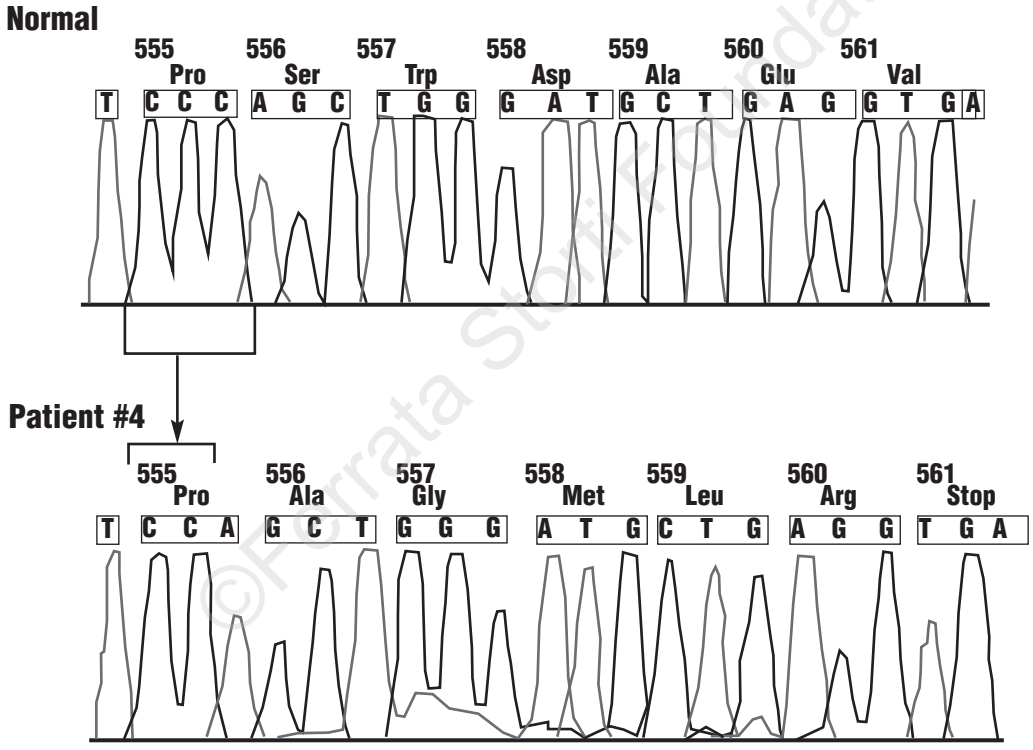

Figure 2. Sequence traces in the region of 1665delC (*V561X*) in patient 4. The figure shows a normal control and a homozygote for 1665delC identified in exon 14. The mutation caused a frame-shift change creating a premature stop codon at the 561st amino acid valine (*V561X*).

Results

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

Variation	*Amino acid*	*Type*	*Exon*	*Region*	*References*
Mutations					
84-88insC	E60X	Frameshift	2	Southern Italy	Roetto *et al.*[14]
515T→A	M172K	Missense	4	Southern Italy	Roetto *et al.*[14]
750C→G	Y250X	Nonsense	6	Sicily	Camaschella *et al.*[13]
					Roetto *et al.*[14]
				Italy	Piperno *et al.*[21]
1780-91del	AVAQ594-7del	Deletion	16	Northern Italy	Girelli *et al.*[16]
				Japan	Hattori *et al.*[18]
2069A→C	Q690P	Missense	17	Portugal	Mattman *et al.*[15]
1469T→G	L490R	Missense	11	Japan	ours
1665delC	V561X	Deletion	14	Japan	ours
Probably polymorphisms					
1391G→A	R455Q	Missense	10	Asia	Hofmann *et al.*[22]
64G→T	V22I	Missense	2	China	Biasiotto *et al.*[23]
714C→G	I238M	Missense	5	Japan	Lee *et al.*[24]
					Mattman *et al.*[15]
					ours

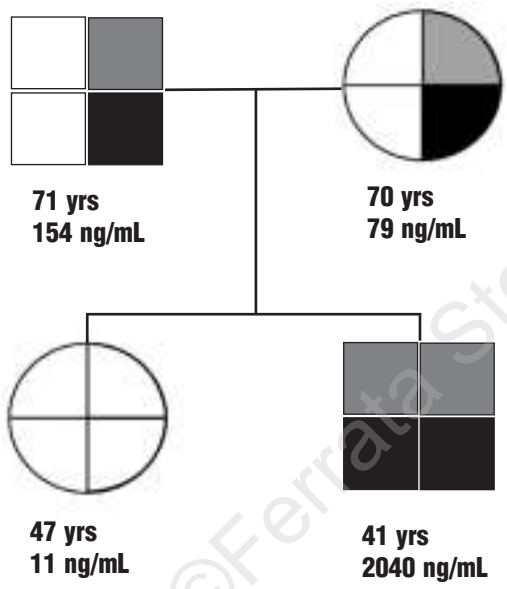

Figure 3. The pedigree of patient 7 with *I238M* and *L490R*. The age and serum ferritin concentration of the patient and family members are shown in the panel. The serum ferritin concentration was determined at the first medical examination for the patient and 5 years later for the family members. The aged parents were compound heterozygotes for the mutations and not affected by iron overload, as shown by normal ferritin levels. Provided that *L490R* is responsible for the iron overload of autosomal recessive inheritance, *I238M* is a polymorphism as reported.[24]

(L490R). 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 hemochromatosisis 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.[19] One family of 4 affected members with a novel mutation of the H chain of ferritin was reported from the northeast island of Hokkaido.[20] In a previous study, we reported an AVAQ 594-597 deletion of *TFR2* in a hemochromatosis family of the main island.[18] 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.[7,14-18,21-24] 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*

al.[24] 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.[18] 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 our 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,[18] 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.

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