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Identification of New Mutations of the *HFE*, Hepcidin, and Transferrin Receptor 2 Genes by Denaturing HPLC Analysis of Individuals with Biochemical Indications of Iron Overload

Giorgio Biasiotto,* Silvana Belloli, Giuseppina Ruggeri, Isabella Zanella, Gianmario Gerardi, Marcella Corrado, Elena Gobbi, Alberto Albertini, and Paolo Arosio

Background: **Hereditary hemochromatosis is a recessive disorder characterized by iron accumulation in parenchymal cells, followed by organ damage and failure. The disorder is mainly attributable to the C282Y and H63D mutations in the *HFE* gene, but additional mutations in the *HFE*, transferrin receptor 2 (*TfR2*), and hepcidin genes have been reported. The copresence of mutations in different genes may explain the phenotypic heterogeneity of the disorder and its variable penetrance.**

Methods: **We used denaturing HPLC (DHPLC) for rapid DNA scanning of the *HFE* (exons 2, 3, and 4), hepcidin, and *TfR2* (exons 2, 4 and 6) genes in a cohort of 657 individuals with altered indicators of iron status.**

Results: **DHPLC identification of C282Y and H63D *HFE* alleles was in perfect agreement with the restriction endonuclease assay. Fourteen DNA samples were heterozygous for the *HFE* S65C mutation. In addition, we found novel mutations: two in *HFE* (R66C in exon 2 and R224G in exon 4), one in the hepcidin gene (G71D), and one in *TfR2* (V22I), plus several intronic or silent substitutions. Six of the seven individuals with hepcidin or *TfR2* coding mutations carried also *HFE* C282Y or S65C mutations.**

Conclusion: **DHPLC is an efficient method for mutational screening for the genes involved in hereditary hemochromatosis and for the study of their copresence.**

Hereditary hemochromatosis (HH)[1] is a common autosomal recessive disorder of iron metabolism with a prevalence of 1 in 200–300 individuals from Northern Europe *(1, 2)*. The disorder is characterized by increased duodenal iron absorption, which leads to excessive iron deposition in the parenchymal cells of the liver and major organs. If untreated, hemochromatosis can lead to cirrhosis, increased susceptibility to hepatocarcinoma, diabetes, cardiac failure, endocrine complications, and arthritis. The major gene associated with HH was identified in 1996 with the finding of two mutations in the *HFE* gene (OMIM 235200): the 845G>A transition, which codes for the mutation C282Y, and the 187C>G transversion, which codes for H63D *(3)*. Most HH probands are homozygous C282Y or compound heterozygous C282Y/H63D *(3)*. In Southern Europe, a large proportion of individuals (35–45%) with HH phenotypes are neither C282Y homozygous nor C282Y/H63D heterozygous *(2)*; in fact, the prevalence of the C282Y allele decreases from north to south in Europe *(4)*. Several other *HFE* mutations have been identified since 1996, but with rare exceptions they occur together with C282Y and their roles in HH are variable *(5, 6)*.

More recently, other genetic forms of the disorder have been identified. One involves homozygous mutations of the transferrin receptor 2 gene (*TfR2*; OMIM 604720) on chromosome 7. Five Italian *(7–9)* and one Portuguese pedigree *(10)* have been described, carrying five distinct causative mutations (E60X, M172K, Y250X, AVAQdel, and Q690P). Other nucleotide substitutions in the gene have been observed frequently in association with *HFE*

Dipartimento Materno Infantile e Tecnologie Biomediche, University of Brescia, viale Europa 11, 25123 Brescia, Italy.

*Author for correspondence. Fax 39-030-307-251; e-mail arosio@med.unibs.it.

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[1] Nonstandard abbreviations: HH, hereditary hemochromatosis; TfR2, transferrin receptor 2; and DHPLC, denaturing HPLC.

mutations *(10, 11)*, but their frequencies in HH remain unclear. TfR2 has 48% identity to transferrin receptor (TfR1) in the extracellular portion of the protein and shows affinity binding to transferrin *(12)* but not to HFE *(13)*, and its physiologic role is unclear at present.

Another form of HH, with earlier symptomatic presentation and more severe clinical course, was found associated with rare homozygous disabling mutations of the hepcidin gene (OMIM 606464) on chromosome 19 *(14)*. Two Mediterranean pedigrees have been described that carry the mutations R56X and 93delG. Hepcidin is a small peptide expressed in liver with antimicrobial activity, and is thought to be a key regulator of iron absorption in mammals *(15, 16)*. To date, extensive analyses of the hepcidin gene in HH have not been reported.

Two other forms of HH have been described. A juvenile HH has been associated with a gene on chromosome 1, which has not yet been identified *(17)*. Finally, an atypical form of HH with dominant transmission is associated with the ferroportin 1 gene on chromosome 2 (OMIM 604353). Up to eight causative mutations have been described *(18–25)*. The gene encodes a membrane protein with a putative role in iron efflux *(26)*.

The products of the genes involved in these HH variants possibly participate in a common regulatory pathway, and the copresence of different mutations might explain the variable clinical and biochemical penetrance of *HFE* HH *(27)*.

Denaturing HPLC (DHPLC) allows rapid DNA scanning *(28)* and has already been applied to the study of the *HFE (29)*, ferritin *(30)*, and ferroportin 1 genes *(20)*. It shows promise as a suitable technique for studying the genetic basis of HH and iron overload. To verify this hypothesis, we used DHPLC to screen for mutations in the *HFE* (exons 2, 3, and 4), hepcidin (all three exons), and *TfR2* (exons 2, 4, and 6) genes in a cohort of 657 individuals with biochemical signs of iron overload.

Materials and Methods

PATIENTS

Blood samples were obtained after informed consent from 657 individuals who underwent *HFE* genotyping for diagnosis of HH and had high ferritin concentrations (>400 μg/L) or transferrin saturation (>50%). All were all from Northern Italy, the region around Brescia. *HFE* C282Y and H63D mutations were detected by endonuclease digestion *(31)* before the study.

PCR

Genomic DNA was extracted from 200 μL of EDTA-anticoagulated blood with use of the QIAamp DNA Blood Mini Kit and according to the manufacturer's instructions (Qiagen). Primers for PCR amplifications (MWG Biotech) were designed to amplify the exons and flanking regions and are listed in Table 1. PCR reactions were carried out in a 50-μL volume with 100 ng of template genomic DNA, 200 μM each of the deoxynucleotide triphosphates, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 1.5 U of AmpliTaq Gold (Applied Biosystems), and 25 pmol of each primer. For hepcidin exon 2/3, we used 2.5 mM $MgCl_2$. The PCR annealing temperatures and conditions are summarized in Table 1.

DHPLC

Heteroduplexes were obtained by denaturing the PCR products at 94 °C for 10 min and cooling at 56 °C for 60 min. Mutation analysis was performed according to a previously described method *(28–30)* on a Transgenomic WAVE® System equipped with a preheated C_{18} reversed-phase column based on nonporous poly(styrene/divinylbenzene) particles (DNASep™; Transgenomics). We injected 8 μL of the PCR mixture into the column, and the heteroduplexes and homoduplexes were eluted with a linear gradient formed by mixing buffer A (0.1 mol/L triethylamine acetate, pH 7.0) and buffer B (0.1 mol/L

Table 1. PCR and DHPLC conditions.

Gene	Exons	Primers, 5′–3′	PCR temperature (no. of cycles)	Amplicon, bp	DHPLC temperature, °C
Hepcidin	1	1F: GGAAATGAGACAGAGCAAAGG	57 °C (40)	243	63.9
		1R: GAGACGTCCTGAGCTCTGCT			
Hepcidin	2/3	2F: ATCCTCTGCACCCCCTTCTGC	64 °C (35)	432	63.1
		3R: CTCTTAGCACAGACACTCGGC			
HFE	2	2F: GGTGTGTGGAGCCTCAACAT	59 °C (5)	334	61.1
		2R: GGAAAATCACAACCACAGCAAGG	57 °C (30)		
HFE	3	3F: GGACCTATTCCTTTGGTTGCA	59 °C (5)	371	63.5
		3R: TCCACTCTGCCACTAGAGTA	57 °C (30)		
HFE	4	4F AGTTCCAGTCTTCCTGGCAA	59 °C (5)	368	62.4
		4R: AGCTCCTGGCTCTCATCAGT	57 °C (30)		
TfR2	2	2F: CCTCATTATTGCCAGATGCC	59 °C (5)	406	64.2
		2R: TCTCTTTATGCCCACCTCTG	57 °C (30)		
TfR2	4	4F: TCTGGCATCCTTCCCTCTTC	59 °C (5)	213	65.8
		4R: ACGGATCCGGGAATTGCAG	57 °C (30)		
TfR2	6	4F: GCACGAGCCCTTTCCTGG	56 °C (35)	269	67.5
		4R: ACCCTGAACGATTCTCACTG			

triethylamine acetate, pH 7.0, containing 250 mL/L acetonitrile) at a constant flow rate of 0.9 mL/min. DNA was detected by monitoring the absorbance at 260 nm. For each fragment, the initial and final concentrations of buffer B were adjusted to obtain a retention time between 3 and 5 min. The column was then washed with 100% buffer B for 30 s and equilibrated at starting conditions for 1 min. The melting characteristics of the DNA fragments were predicted by use of the Wavemaker™ software.

SEQUENCE ANALYSIS

Samples with altered elution profiles in DHPLC analysis were sequenced directly with the appropriate primers.

Results

DHPLC was used to analyze a DNA from 657 individuals with abnormal biochemical indicators of iron status (serum ferritin >400 μg/L or transferrin saturation >50%). Before the study, DNA from these individuals was analyzed for the common C282Y and H63D mutations by endonuclease digestion *(31)*.

HFE ANALYSIS AND MUTATIONS

For DHPLC analysis of the *HFE* gene, we considered exons 2 and 4, which contain the H63D and C282Y mutations, respectively, and exon 3, in which six substitutions have been identified to date *(6)*. Exons 1, 5, and 6 were excluded from the study because only one coding mutation (R330M) has been identified in them *(6)*. We first applied the PCR primers and DHPLC conditions described by DeValia et al. *(25)*, but we found a high proportion (~50%) of abnormal DHPLC patterns after ~100 analyses of *HFE* exon 2. DNA sequencing revealed that they all contained a common polymorphism in intron 3 (IVS3 + 4T>C), which was included in the amplicon. Because this complicated the interpretation of DHPLC profiles, we designed a new reverse primer that excluded the polymorphic site (Table 1). All abnormal DHPLC profiles obtained with this amplicon were found to be associated with DNA variations, as listed in Tables 2 and 3. The characteristic elution profiles of H63D and C282Y mutations (Figs. 1 and 2) were always associated with the heterozygous samples identified by endonuclease digestion; therefore, DNA sequencing of these samples was performed only in the first 10 samples. However, DHPLC does not recognize mutant homozygous in a single run because the altered profile is attributable to the differential melting of heteroduplexes. Therefore, to detect homozygous mutations, the normal amplicons must be mixed with an equal amount of wild-type DNA, denatured, reannealed, and analyzed for heteroduplexes. For faster identification of C282Y and H63D homozygotes, the exon 4 and 2 amplicons with wild-type DHPLC profiles were pooled in groups of four, denatured, reannealed, and analyzed again by DHPLC. An abnormal profile indicated the presence of a homozygote in the pool, which was then identified by mixing the samples singularly with wild-type DNA and analyzing them again.

Table 2. Known *HFE* genotypes in the individuals with altered iron status.

Mutation	Individuals with genotype		Allelic frequency, %
	n	%	
H63D+/+	23	3.5	16
H63D+/−	164	24.9	
C282Y+/+	36	5.5	13.7
C282Y+/−	108	16.4	
S65C+/−	14	2.1	1.07
H63D+/− and S65C+/−	2	0.3	−[a]
H63D+/− and C282Y+/−	22	3.3	−[a]
S65C+/− and C282Y+/−	2	0.3	−[a]

[a] The allelic frequency could not be determined because of the low numbers of individuals with this mutation combination.

All heterozygous and homozygous H63D and C282Y mutations identified by enzymatic digestion were recognized by DHPLC analysis with no false-positive or -negative results. This indicated 100% sensitivity and demonstrated the reliability of this assay, as reported previously *(29)*. The S65C mutation was found in 14 samples, and this was the only other known *HFE* mutation found in the cohort. In addition, we identified two new DNA variations in exon 2: 196C>T, which encodes for the substitution Arg^{66}>Cys (Fig. 1A), and 88C>T, within the Leu 30 codon, which does not cause an amino acid substitution. In exon 4 we found three new substitutions: two silent ones (696C> T in codon Pro232 and 867C>G in codon Leu289) and one, 671G>A, that encodes for the change of Arg^{224} to Gly (Fig. 2). Finally, we found one substitution in intron 3: IVS3 + 21T>C (DHPLC profile not shown). All of the newly identified sequence changes are listed in Table 3. A summary of the frequencies of C282Y, H63D, and S65C homo- and heterozygous samples is shown in Table 2.

TfR2 ANALYSIS AND MUTATIONS

The gene *TfR2* is composed of 18 exons. As an initial study for DNA variations in this gene, we considered only exons 2, 4, and 6, which harbor the causative mutations E60X, M172K, and Y250X, respectively *(7, 8)*. For our initial DHPLC analyses of the amplicons, we used the temperatures suggested by the WAVE DNA Fragment Analysis System (Transgenomic). The analytical run temperature was then confirmed after we found the mutations. The final conditions are listed in Table 1. All samples with abnormal DHPLC profiles were subjected to DNA sequencing for localization of the mutation. Because of the low frequency of DNA variations, we did not search for homozygous mutations in the gene. No DNA variation was found in the exon 4 amplicon. In the exon 2

Table 3. Novel DNA variations in the *HFE*, hepcidin, and *TfR2* genes in the individuals with altered iron status.

Gene	Amplicon	Substitution	Amino acid change	No. of cases	Copresence with *HFE* mutations, n (mutation)
Coding mutations					
HFE	Exon 2	196C>T	R66C	1	
	Exon 4	673G>A	R224G	1	
Hepcidin	Exons 2/3	212G>A	G71D	4	2: C282Y/−[a]
					2: C282Y/C282Y[a]
TfR2	Exon 2	64G>T	V22I	3	1: C282Y/C282Y
					1: S65C/−
					1: Wild type
Silent coding variations and intronic polymorphisms					
HFE	Exon 2	88C>T	L30L	1	H63D/−
	Exon 4	696C>T	P232P	1	H63D/−
	Exon 4	867G>C	L289L	1	
	Exon 3	IVS3 + 21T>C		1	
Hepcidin	Exons 2/3	IVS2 + 7G>A		2	1: C282Y/−
					1: C282Y/− and H63D/−
	Exons 2/3	IVS2 + 56G>A		1	1: C282Y/−
TfR2	Exon 2	IVS3 + 10G>A		1	1: Wild type
	Exon 2	IVS3 + 15C>T		2	2: Wild type
	Exon 6	IVS5-9T>A		9	5: C282Y/C282Y[b]
					3: C282Y/−[b]
					1: C282Y/− and H63D/−
	Exon 6	IVS5-24G>C		2	1: H63D/−
					1: Wild type

[a] Siblings.
[b] Siblings in a large family.

amplicon, we identified three new DNA variations: 64G>T, which encodes for the substitution Val^{22}→Ile, was present in three samples; IVS3 + 10G>A, in intron 3, was present in one sample; and IVS3 + 15C>T, also in intron 3, was present in two samples (Fig. 3). In the exon 6 amplicon we found two intronic substitutions: IVS5-9T>A in nine samples, eight of which were from members of the same family; and IVS5-24G>C in two samples.

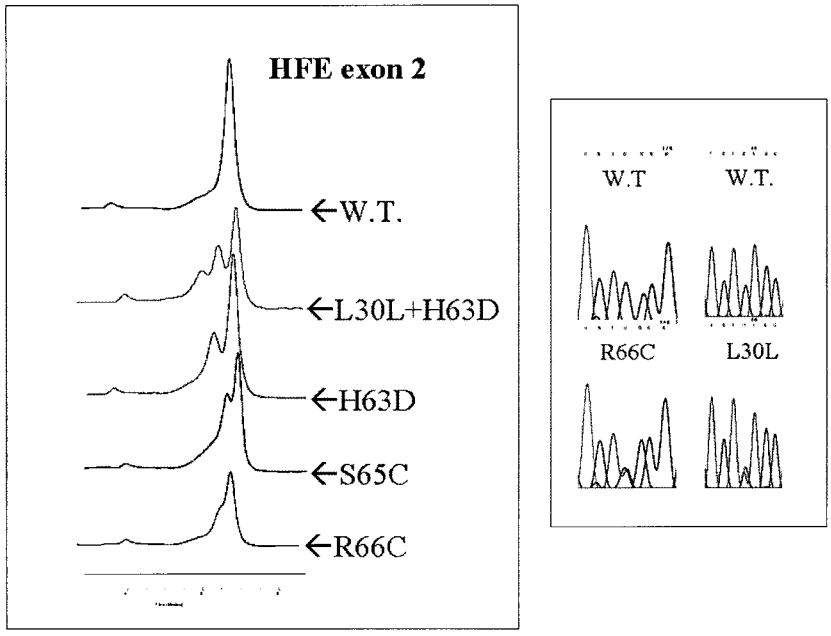

Fig. 1. DHPLC elution profiles and DNA sequencing of the identified mutants in the *HFE* amplicon for exon 2.

The DNA samples were run at the near-denaturing temperature of 61.1 °C. Wild-type (*W.T.*) samples eluted as single symmetric peaks corresponding to the homoduplex, and the heterozygous mutant samples showed extra components eluting ahead the wild-type peak that corresponded to the partially melted heteroduplexes. The mutations show different elution profiles.

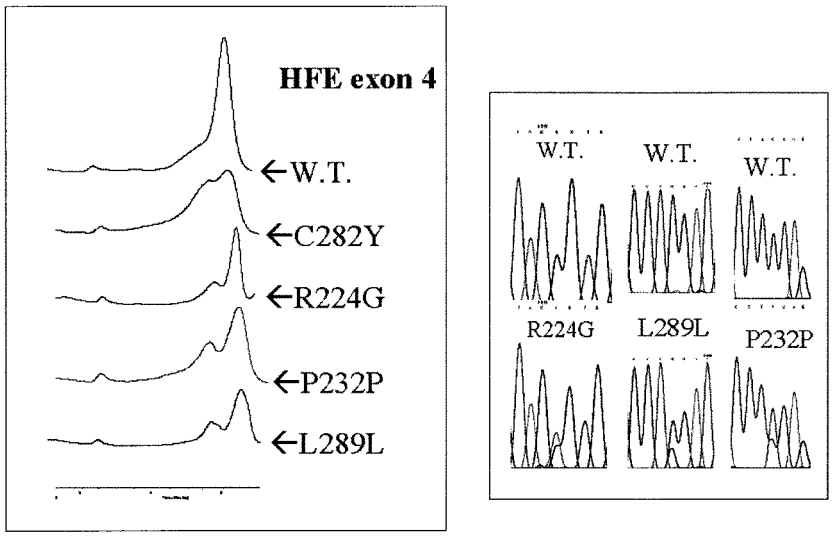

Fig. 2. DHPLC elution profiles and DNA sequencing of the identified mutants in the *HFE* amplicon for exon 4.

Samples were run at the analytical temperature of 62.4 °C. *W.T.*, wild type.

HEPCIDIN ANALYSIS AND MUTATIONS

The hepcidin gene contains three exons, which were amplified in two amplicons. The first was for exon 1, which encodes for the leader sequence, and the second for exons 2 and 3. The primers, PCR conditions, and DHPLC analytical temperatures for the two amplicons are listed in Table 2. The availability of a mutated sample (R56X; kindly provided by Dr. Camaschella, Torino, Italy) facilitated the validation of DHPLC conditions for the amplicon of exons 2 and 3. In this amplicon, we identified the 212G>A variation, which encodes for the $Gly^{71}\rightarrow Asp$ substitution, in four individuals. In addition we found two other substitutions in intron 2: IVS2 + 7G>A (two samples) and IVS2 + 56G>A (one sample). No DNA variations were found in the amplicon of exon 1. The DNA variations are listed in Table 3, and the corresponding DHPLC profiles and DNA sequences are shown in Fig. 4.

COPRESENCE OF HEPCIDIN/*TfR2* WITH *HFE* MUTATIONS

We noticed an unusually high frequency of *HFE* mutant alleles in the individuals with hepcidin or *TfR2* mutations. For example, all four individuals who were hepcidin G71D+/− carried the *HFE* C282Y allele. Of the three individuals who were *TfR2* V22I+/−, one was C282Y+/−, one was H63D+/−, and one was wild-type *HFE*. In addition, 9 of the 14 unrelated individuals with DNA variations in the hepcidin or *TfR2* genes carried the *HFE* C282Y allele, and 1 was S65C+/− (Table 3).

Discussion

We used DHPLC to analyze DNA variations in the *HFE*, hepcidin, and *TfR2* genes in 657 individuals who presented for *HFE* genotyping and had altered biochemical indicators of iron status. The results are summarized in Tables 2 and 3. This population had already been evaluated for mutations in the 5′-untranslated regions of the genes for H- and L-ferritin to determine the genetic causes of hyperferritinemia *(29)*.

HFE MUTATIONS

Our data confirm a perfect match between DHPLC and endonuclease digestion for identification of the C282Y and H63D mutations, both homo- and heterozygous *(29)*. Of the other described *HFE* mutations, we found only one, S65C, in 14 samples. The allelic frequencies of these three clinically important mutations (16% for H63D, 13.7% for C282Y, and 1% for S65C) were comparable to

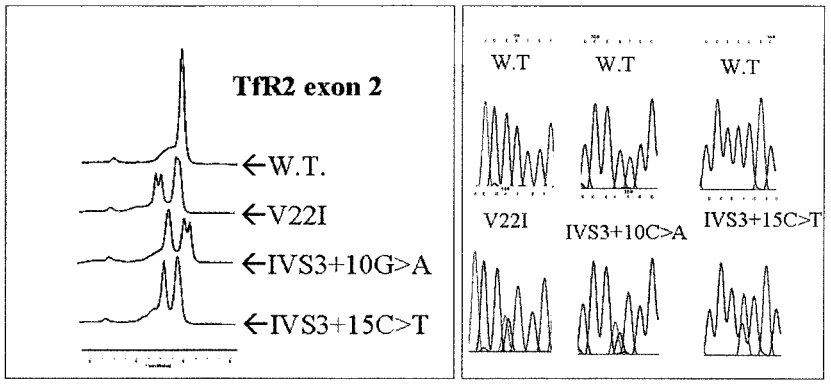

Fig. 3. DHPLC elution profiles and DNA sequencing of the identified mutants in the *TfR2* amplicon for exon 2 at the temperature of 64.2 °C.

W.T., wild type.

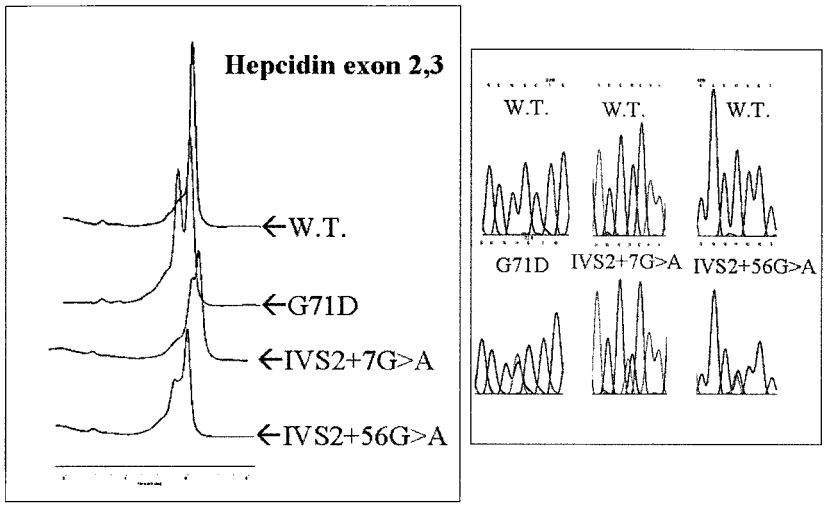

Fig. 4. DHPLC elution profiles and DNA sequencing of the identified mutants in the hepcidin amplicon for exons 2 and 3 at the temperature of 63.1 °C.
W.T., wild type.

those in another group of North Italian individuals with altered iron status *(32)* and show an enrichment of C282Y allele frequency of approximately sixfold compared with unselected blood donors *(33, 34)*. We found six new *HFE* substitutions, four of which can be considered silent polymorphisms because they do not cause amino acid modification (88C>T, 696C>T, and 867G>C) or are intronic and do not appear to introduce alternative splicing (IVS3 + 21T>C). The remaining two caused amino acid changes. Arg^{66}→Cys is located next to Ser^{65} and modifies a residue exposed on the molecule surface that may be involved in TfR1 binding; the mutation thus might have functional relevance. Arg^{224}→Gly modifies a residue located on a β-strand and exposed to solvent, and thus might affect affinity binding to β_2-microglobulin. The mutations were all heterozygous, and the individuals carrying them (71 years of age and hepatitis B surface antigen positive, and 61 years of age and hepatitis C positive, respectively) showed serum ferritin values marginally above the reference interval (377 and 340 μg/L, respectively), which may be related to viral hepatitis.

TfR2 mutations

The *TfR2* gene has been studied by exonic DNA sequencing in 89 individuals, 15 of whom had altered iron status *(10)*. The study identified the HH causative homozygous Q690P mutation, four heterozygous coding mutations (A75V, I128M, A376A, and R752H), and various polymorphisms. Our DHPLC analysis was applied to a larger population and considered only 3 of the 18 exons. It identified three new intronic variations and IVS5-9T>A, which has been already reported *(10)*. More interesting, we found a new coding mutation that causes the substitution Val^{22}→Ile. Val^{22} is in the cytoplasmic domain of the protein next to a putative endocytosis signal (residues 23–26); therefore, the mutation might affect TfR2 cycling. The only individual carrying the mutant allele without *HFE* mutations (72 years of age and hepatitis B surface antigen positive) had a serum ferritin value of 415 μg/L, possibly related to viral hepatitis.

Hepcidin mutations

This is the first reported use of DHPLC for scanning of the hepcidin gene in a large population. The three identified DNA variations were in amplicon 2, which includes the sequence encoding the mature portion of the protein. Two of the variations were intronic and do not seem to affect splicing. The only coding mutation, G71D, modifies Gly^{71}, which is located in between two pairs of cysteines with important structural roles *(15)*. However, Gly^{71} is not conserved in the mouse, rat, or fishes, being substituted with basic or neutral Arg, Asn, or Gln *(35)*, and the functional effect of the mutation cannot be predicted. It introduces a negative charge in a basic region of the molecule containing Lys, Arg, and His residues, which might affect interactions with its putative ligands, might stabilize the molecule by the formation of salt bridges, or might modify its antimicrobial activity. The four individuals carrying the allele belonged to two families form the same geographic area (Valtrompia, near Brescia), and all had *HFE* mutations.

Copresence of hepcidin/*TfR2* variations and *HFE* mutations

A curious observation is that most of the individuals with hepcidin (5 unrelated) or *TfR2* mutations (10 unrelated) also carried the *HFE* C282Y allele. In fact, the C282Y allele was present in 60% of the unrelated individuals with DNA variations in the hepcidin or *TfR2* genes and only in 22% of the total population we analyzed. This is unusual because most of the mutations are silent polymorphisms. However, the numbers are too small for statistical analysis. More interesting is the finding that six of the seven individuals with coding mutations (i.e., hepcidin G71D and *TfR2* V22I) carried the *HFE* C282Y or S65C allele (Table 3). In addition, Mattman et al. *(10)* reported that 7

of 10 individuals with coding mutations in the *TfR2* gene carried the *HFE* H63D allele, and Hofmann et al. *(11)* found the *TfR2* R455Q mutation in a family with the *HFE* C282Y allele. This apparent nonrandom copresence of mutations of the hepcidin, *TfR2*, and *HFE* genes, which are located in different chromosomes, might imply functional relationships. To explore this possibility, we analyzed the families. One family with the G71D allele had three siblings who were *HFE* C282Y+/+. The male (G71D+/−; 48 years of age) had serum ferritin and transferrin saturation values of 538 μg/L and 100%, respectively; one female (G71D+/−; 54 years of age) had values of 149 μg/L and 98%; and the other female (G71D−/−; 43 years of age) had comparable values of 293 μg/L and 93%. Thus, the G71D allele did seem to have evident effect of the biochemical indices of iron status within the family. However, all siblings had signs of hepatic disorders (hepatitis B positive), which complicated the clinical pattern. The individuals with the *TfR2* V22I allele were unrelated. One was *HFE* C282Y +/+ (age, 51 years), showed no signs of hepatitis, and had a serum ferritin of 873 μg/L. Another was heterozygous for *HFE* S65C (age, 62 years), had no signs of hepatitis, and had serum ferritin of 1557 μg/L. This rather high value suggests that the mutant allele might contribute to hyperferritinemia.

In conclusion, DHPLC is an efficient method for large population screening of the genes involved in HH, being much faster and more economical than direct exonic DNA sequencing. It detects known and unknown heterozygous DNA variations in single analyses and thus is potentially useful for the identification of new mutations. A minor limitation of the method is that identification of homozygous mutations requires a second run after sample mixing and reannealing. The extension of DHPLC analysis to the unexplored *TfR2* exons and to other genes, such as ferroportin 1, may confirm the observed high frequency of the copresence of mutations of the *HFE* and hepcidin/*TfR2* genes and verify how they modify the penetrance of *HFE* hemochromatosis.

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