

Transferrin Receptor Mutation Analysis in Hereditary Hemochromatosis Patients

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ABSTRACT: The Cys282→Tyr mutation in the HFE gene is carried by the majority of hereditary hemochromatosis patient chromosomes, yet some patients do not seem to harbor any mutation in this gene. This suggests a possibility that these patients may have a mutation in other genes in the same pathway as HFE. We analyzed the cDNA sequences of transferrin receptor (TFR), which was recently shown to interact with HFE, in twenty-one hereditary hemochromatosis patients including sixteen individuals who did not carry a Cys282→Tyr mutation. A nucleotide substitution (424A→G), which resulted in the Ser142→Gly amino acid substitution, was the only amino acid polymorphism detected in the open reading frame of the TFR gene in these patients. This amino acid substitution was a rather common polymorphism in the general population (49%) and its frequency did not significantly differ in the hereditary hemochromatosis (HH) patients regardless of the HFE genotype. Thus, amino acid changes in the TFR gene do not appear to play a role in HH even when the patients do not have a HFE mutation. However, this study does not rule out the possibility of the involvement of mutations in non-coding regions.

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INTRODUCTION

The HFE (formerly HLA-H) gene on chromosome 6p was identified as a strong candidate gene for hereditary hemochromatosis (HH), with its Cys282→Tyr (C282Y) mutation existing in 85% of the patient chromosomes (1). Other researchers confirmed the presence of this mutation in a majority of the HH patient chromosomes analyzed (2-6). Another mutation His63→Asp (H63D), despite its relatively high frequency (17%) in the chromosomes from the general population, was also suggested to be involved in HH, albeit with very low penetrance and only when found in conjunction with C282Y

as a compound heterozygote (1). While 12% of the HH patients did not carry a C282Y mutation on either chromosome, deviation from the Hardy-Weinberg expectations in the C282Y homozygote:heterozygote genotype ratio suggested that the majority of these non-C282Y hemochromatosis cases were not caused by a HFE gene defect (1). This indicated that some HH patients had iron overloading because of factor(s) other than HFE mutations. The question arises: Is there a mutation in another gene, possibly in the same pathway as HFE in those patients, or is it due to a non-genetic (environmental) factor?

A study addressing this issue did not find a

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mutation in the genes for a HFE binding protein (β 2-microglobulin) and a potential HFE interacting protein (calreticulin) (7). Our recent biochemical work has identified an interaction between HFE and transferrin receptor (TFR) (8,9). Moreover, HFE reduces TFR's affinity for transferrin, and both the C282Y and the H63D mutations of HFE diminish this functional activity of HFE (8). This result implies HFE modulates the TFR pathway and that TFR is involved in iron overloading in HH patients containing the HFE mutations. This raises the possibility that a mutation in TFR may cause a similar phenotype to that of an HFE mutation, for example by destroying the interaction between TFR and HFE. The TFR gene has been mapped to chromosome 3 (10-12). Thus, it was not originally considered as a candidate for the HH gene, since the HH locus had already been mapped to chromosome 6p (13,14). However, the apparent presence of HH patients that did not contain mutations in the HFE gene, revived the possibility that a mutation in another gene involved in iron homeostasis, such as TFR, could be implicated in the etiology of HH. We tested this hypothesis by examining the DNA sequence of the TFR coding region in HH patients.

MATERIALS AND METHODS

Patient Materials

Diagnosis criteria for the HH patients, preparation of genomic DNA and first strand cDNA from lymphoblastoid cell lines were as described (1). All the HH patients and control individuals were Caucasians. All patient DNAs were previously genotyped for C282Y and H63D mutations in the HFE gene (1).

RT-PCR and DNA Sequence Analysis

DNA sequence analysis of the TFR gene were performed on the reverse transcription PCR (RT-PCR) products using the first-strand cDNAs from patient lymphoblastoid cells as templates (1).

The TFR cDNA sequence (15,16) was split into three amplicons for RT-PCR. The PCR reactions were done using the Expand™ PCR kit (Boehringer Mannheim) with cycles of: [94C for 20 sec, 60C for 30 sec, 72C for 1 min, 35 cycles] with external primers, followed by [94C 20 sec, 60C 30 sec, 72C 1min, 25 cycles] using internal primers. The DNA sequences of the PCR primers were: TFR.P1 (amplicon 1, forward, external); 5'-CA GAGCGTCGGGATATCG-3', TFR.P2 (amplicon 1, reverse, external); 5'-AGT CTGTTTTCCAGTCAGAGGG-3', TFR.P3 (amplicon 1, forward, internal); 5'-CGCGCTA GTGTTCTTCTGTG-3', TFR.P4 (amplicon 1, reverse, internal); 5'-GCAATCCTGATGACC GAGAT-3', TFR.P11 (amplicon 2, forward, external); 5'-TGATTGTCAGAGCAGGGAAA-3', TFR.P12 (amplicon 2, reverse, external); 5'-GCTCTGTATTGGTTCAGATCCC-3', TFR.P13 (amplicon 2, forward, internal); 5'-AGCAGGGA AAATCACGTTTG-3', TFR.P14 (amplicon 2, reverse, internal); 5'-GGTTCAATTCAAC ATCATGGG-3', TFR.P21 (amplicon 3, forward, external); 5'-AATGCAAAATGTGAA GCATCC-3', TFR.P22 (amplicon 3, reverse, external); 5'-GGAGAAGGTCTTTC AACCTGG-3', TFR.P23 (amplicon 3, forward, internal); 5'-GCTGCTTCCCTTTCCTTG-3', TFR.P24 (amplicon 3, reverse, internal); 5'-AGGAAAGA GGCAGTCCCAT-3'. PCR products were purified using a Qiaquick kit (Qiagen) and DNA sequencing was performed on Applied Biosystems 373 and 377 DNA sequencers using dye terminator reactions. Analysis of DNA sequence variations was done using the Staden sequence analysis package (17).

Genotyping by Oligonucleotide Ligation Assay (OLA)

The TFR 424A→G polymorphism was genotyped by OLA (18). The PCR primers used to amplify the polymorphic region were TFR.d1.P3 (forward); 5'-TTCATT CAGCAGC TTGATGG-3', TFR.d1.P6 (reverse); 5'-TAAC AAGCCATTCCCCAC-3'. The oligonucleotides

used for OLA were: TFR.d1.A (5'-side oligonucleotide for A-allele); 5'-biotin-GGACA GCACAGACTTCACCA-3', TFR.d1.G (5'-side oligonucleotide for G allele); 5'-biotin-GACAGC ACAGACTTCACCG-3', and TFR.d1.X (3'-side oligonucleotide); 5'-pGCACCATCAAGTGCT GAAT. All the oligonucleotides were synthesized by Genset.

RESULTS AND DISCUSSION

Overlapping RT-PCR amplicons were designed to cover the entire protein-coding region of the TFR gene and were amplified from lymphoblastoid first-strand cDNAs from twenty-three HH patients. These RT-PCR products were analyzed by DNA sequencing. HFE genotypes of these HH patients were two C282Y homozygotes, five C282Y heterozygotes (including three H63D compound heterozygotes), and sixteen patients lacking C282Y (seven of which were H63D

heterozygotes). This group of patients was chosen to consist mainly of non-C282Y HFE genotype, because those were the patients suspected to have an iron overload because of a reason other than a HFE gene defect.

In the DNA sequence analysis, two nucleotide substitutions in the TFR gene-coding region were detected in these patients. The first was a 789G→C silent (no amino acid change) polymorphism. This variation was also detected in a comparison of the two published TFR cDNA sequences (15,16). The second nucleotide substitution 424A→G causes a Ser142→Gly (S142G) amino acid change. 424A→G has been recognized as a sequence difference between genomic PCR products and a cDNA clone in GenBank (19).

Patients and controls were genotyped for the 424A→G polymorphism using the oligonucleotide ligation assay (OLA) method.

Table 1. Frequency of the TFR 424A→G (S142G) polymorphism in the hereditary hemochromatosis patient and CEPH control chromosomes

Genotype of HFE	HH status (number of chromosomes)	A allele	G allele
C282Y homozygote	patients (n=138)	76 (55%)	62 (45%)
	controls (n=0)	0	0
C282Y heterozygote	patients (n=18)	9 (56%)	9 (44%)
	controls (n=4)	2 (50%)	2 (50%)
non-C282Y	patients (n=38)	24 (63%)	14 (37%)
	controls (n=86)	44 (51%)	42 (49%)

The genotyping result of the 424A→G polymorphism in HH patients and CEPH unaffected controls is shown in Table 1. In the CEPH controls, we observed 49% frequency of the 424G allele (44/90 chromosomes [49%] overall, 2/4 chromosomes [50%] in C282Y heterozygotes and 42/86 chromosomes [49%] from individuals lacking C282Y). The frequency did not vary significantly for any of the subsets of HH patients (62/138 chromosomes [45%] in C282Y homozygotes [$\chi^2=0.34$], 9/18 chromosomes [50%] in C282Y heterozygotes [$\chi^2=0.04$], and 14/38 chromosomes [37%] in non-C282Y patients [$\chi^2=1.56$]). Thus this polymorphism does not appear to be associated with hereditary hemochromatosis, regardless of the patients' HFE genotype. The Ser142 residue is not conserved in mice (20), suggesting it may not be essential for TFR function. The high prevalence of this allele in the general population makes this amino acid substitution unlikely to be deleterious. Since the Ser142→Gly is the only TFR coding change found in these HH patients, and no significant association is seen between either the Ser or the Gly allele and the disease, especially in the non-C282Y patients, a major role of TFR coding mutations in these patients is unlikely. However we could not rule out the possibility that non-coding mutations affecting transcription, splicing or mRNA stability exist.

In conclusion, we did not find evidence for the involvement of the TFR gene in hereditary hemochromatosis, even though its gene product is known to be modulated by an interaction with HFE, making it a good candidate for a potential second HH gene. Thus, the cause of iron overload in non-C282Y patients remains unknown. It should be noted that all of the patients in that study met the following strict diagnostic criteria. They are; the absence of a known cause of secondary iron overload, and the presence of at least two of the following criteria: a) Hepatic iron concentration (HIC) > 4,500 $\mu\text{g/g}$ dry weight; b) calculated hepatic iron index (HII>2.0); c) increased stainable iron in the liver (Perl's Prussian blue stain, grade 3+ or 4+); and

d) >4 g of iron removed by quantitative phlebotomy (>16 units of blood). In addition, all the patients had undergone liver biopsy (1). It remains to be answered whether these patients have an iron overload due to mutations in a yet undiscovered gene, or due to non-genetic causes.

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