

## The Effect of Transferrin Polymorphisms on Iron Metabolism

Submitted 12/24/99

(Communicated by Ernest Beutler, M.D., 12/24/99)

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**ABSTRACT:** The effect of five different transferrin variants (TFv1, TFv2, TFv3, TFv4, and TFv5) on the hemoglobin level, mean corpuscular volume (MCV), ferritin level, percent transferrin saturation (%TS), and the unsaturated iron binding capacity (UIBC) was investigated in subjects with defined *HFE* haplotypes, 919 persons undergoing health screening and 113 patients with clinical hemochromatosis. The most common variant is TFv4; the population distribution of this variant was also studied. None of the variants were found to have an effect on any of the parameters of iron metabolism that were investigated. Moreover, the frequency of these variants in patients with clinically significant hemochromatosis was no different from that in the general population. We conclude that these polymorphisms in transferrin do not play a role in the expression of hemochromatosis, nor do they produce any other significant changes in iron metabolism.

**Keywords:** hemochromatosis, ferritin, *HFE*

### INTRODUCTION

In 1935 Sheldon suggested that "idiopathic" hemochromatosis was a hereditary disease. A number of genes were considered as candidates for the causation of this disorder, and among these was transferrin. Many genetic variants of this iron transport protein were known and in 1961 Trumbull and Giblett (1) concluded that the electrophoretic variants of this protein were not functionally different from the wild-type transferrin and that abnormalities in transferrin were, therefore, not responsible for hemochromatosis. After it was shown in 1975 by Simon et al. (2) that the disease was linked to the *HLA* locus, interest in candidate genes that were not on chromosome 6 disappeared. Indeed, when the *HLA*-linked gene, now designated *HFE*, was cloned in 1996 (3) it became apparent that most European patients with hemochromatosis were homozygous for a common mutation in this gene. It was also apparent, however, that mutations of *HFE* are not the sole cause of hemochromatosis. First of all, even in northern European patients with hereditary hemochromatosis, among whom the prevalence of *HFE* mutations is the highest, some 15% have no *HFE* mutation, even when the complete coding sequence is examined. Moreover, the clinical expression of the homozygous state varies widely. Many patients who are homozygous

for the *HFE* mutation shown no manifestations of hemochromatosis and, indeed, appear to have normal iron stores. On the other hand, some patients with the disease become massively iron loaded and show substantial degrees of tissue damage. Clearly there are other factors that influence iron absorption.

From this point of view it is useful to examine candidate genes and candidate mutations to determine whether they are associated with any aberrations of iron metabolism or of erythropoiesis. One excellent such candidate is the transferrin receptor, particularly since the *HFE* protein complexes with this receptor (4,5). A single mutation (424A→G) causing a serine 142 → glycine substitution is a known polymorphism of the transferrin receptor and Tsuchihashi et al. (6) failed to find any evidence that the frequency of this mutation was higher either in patients with hemochromatosis either with or without *HFE* mutations than in the general population. Transferrin itself is another attractive candidate.

Transferrin is a highly polymorphic gene in which over 30 different genetic variants are known to exist. In the past, the transferrin variants were identified by differences in electrophoretic mobility. Most transferrin variants appear to be functionally normal. Only one transferrin variant has been identified that exhibited abnormal iron binding

**Table 1.** Transferrin Variants (OMIM 19000.001-.005)

Variant	Name	Mutation	nt. change	Gene frequency	Ethnic origin	Ref.
TFv1	D1	D277G	A→G	0.028-0.045	African	(8)
TFv2	CHI	H300R	A→G	0.006-0.169	Asian	(8)
TFv3	B2	G652E	G→A	0.005-0.007	Caucasian	(8)
TFv4	C2	P570S	C→T	0.108-0.188		(8)
TFv5	Bv	K627E	A→G	nd*	nd*	(9)

Note. \*nd, not determined.

**Table 2.** Primers Used to Amplify Regions Encompassing TFv1-TFv5

TFv	5'-Primer	3' Primer	Size (bp)
1,2	GCAGAGATTTCTTTTCTCTTCAGT	GATTCGGATGGCAGTGACAT	168
4	AACCCTGATCCATGGGCTAAG	GGACGCAAGCTTCCTTATCTT	156
3,5	CACCTATTTGGAAGCAACGTAAGT	ATGAGGTGGAGCATTCTCAGG	189

examined five allelic variants of transferrin. These are summarized in Table 1. We have now investigated the effect of these on the severity of hemochromatosis and on parameters of iron metabolism in a large group of subjects.

## MATERIALS AND METHODS

Two groups of subjects were studied. The first consisted of patients with clinical hemochromatosis with known *HFE* genotypes. These patients were subjects of a previous study (10). The second group of patients comprised predominantly healthy individuals undergoing voluntary health screening in a health appraisal clinic who had volunteered to participate in a study of hemochromatosis. Not all parameters were available for every individual.

*HFE* genotypes were determined by allele-specific oligonucleotide hybridization, as described previously (10). Transferrin genotypes were determined by polymerase chain reaction using the primer sets described in Table 2 to amplify regions of the transferrin gene containing the five known genetic variants.

**Table 3.** ASOH Primers Used to Detect TFv1-TFv5

Variant	Probe	Sequence
TFv1	TF917A	TGGCAAAGACAAATCAA
	TF917G	TGGCAAAGGCAAATCAA
TFv2	TF986A	CTCTGCCCACGGGTTTT
	TF986G	CTCTGCCCACGGGTTTT
TFv3	TF2042A	ATACTTAGAAGAAGAATA
	TF2042G	ATACTTAGGAGAAGAATA
TFv4	TF1795C	CCAGGAAACCTGTGGAG
	TF1795T	CCAGGAAATCTGTGGAG
TFv5	TF1966A	CGGAAACCAAGGACCTT
	TF1966G	CGGAAACCGAGGACCTT

The polymerase chain reaction was performed multiplexing the three primer sets in a single reaction. Essentially, the 50  $\mu$ l reaction mix contained 50 ng of each primer, 250 ng of DNA, 0.1 M Tris HCl pH 8.8, 0.1 M  $(\text{NH}_2)_2 \text{SO}_4$ , 50 mM  $\text{MgCl}_2$ , 4 mg/ml BSA, and 1 U Taq polymerase. After an initial denaturation at 95 C for 2 min, the polymerase chain reaction was performed for 30 cycles at 95 C for 1 min, 61 C for 30 s and 72 C for 45 s. An aliquot of the amplified DNA fragments (5  $\mu$ l) were spotted on 0.45  $\mu$ M nylon membranes (Nytran supercharge 96 well plate) and allele specific oligonucleotide hybridization (ASOH) performed.

ASOH was performed for each transferrin variant. The primers used for ASOH are described in Table 3.

Each oligomer was labeled by polynucleotide kinase using  $\gamma$ -ATP and the membranes hybridized

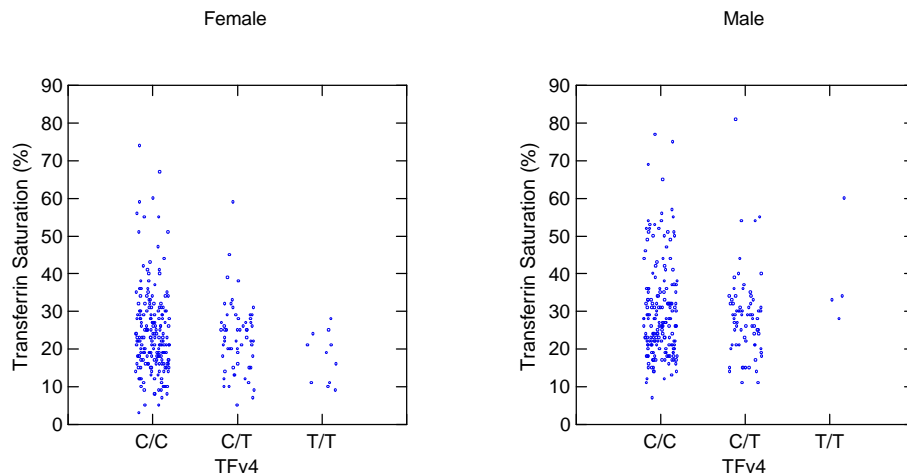
**Table 4.** Ethnic Distribution of the TFv4 Polymorphism in 915 Subjects

Ethnicity	TFv4 Genotype			TFv4 Allelic Frequency	
	C/C	C/T	T/T	C	T
White	500	166	20	0.8499	0.1501
African	27	7	0	0.8971	0.1029
Hispanic	65	14	3	0.8780	0.1220
Asian/Pac. Is.	34	17	3	0.7870	0.2130
Native American	3	1	0	0.8750	0.1250
Mixed or unknown	40	14	1	0.8545	0.1455
TOTAL	669	219	27	0.8508	0.1492

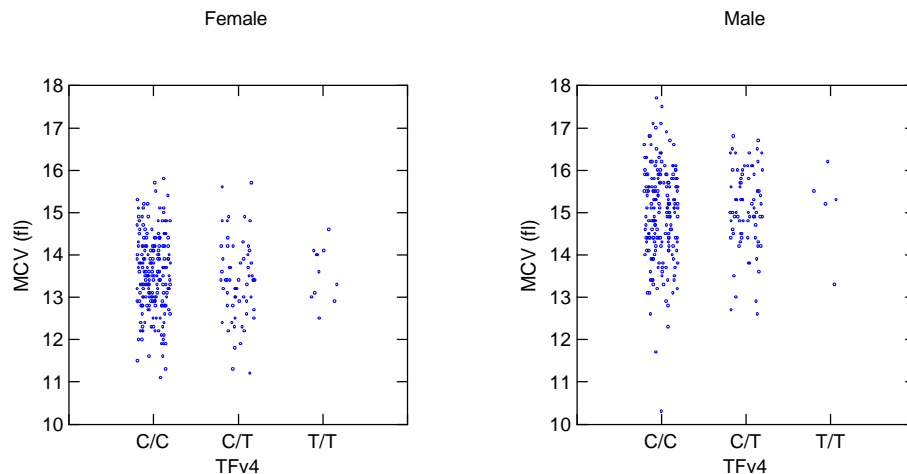
**Table 5.** Effect of TFv4 on the Hemoglobin, Mean Corpuscular Volume (MCV) Transferrin Saturation (%TS), and Unsaturated Iron Binding Capacity (UIBC) of Individuals with the Wildtype *HFE* Genotype

TFv4		Hemoglobin	MCV	Ferritin*	% TS	UIBC	Age
<b>Female</b>							
C/C	Mean	13.5	89.5	52.80	24.4	280.2	55.5
	SE	0.06	0.34	(49.48,56.33)**	0.80	4.82	0.97
	n	202	202	188	198	198	209
C/T	Mean	13.4	90.0	48.35	23.0	282.4	57.2
	SE	0.12	0.46	(43.51,53.72)	1.22	7.03	1.77
	n	61	61		59	59	61
T/T	Mean	13.6	88.2	37.43	17.7	288.7	58.3
	SE	0.20	0.93	(29.03,48.25)	2.02	13.42	4.87
	n	11	11	11	11	11	11
<b>Male</b>							
C/C	Mean	15.0	90.5	119.42	29.6	240.1	56.5
	SE	0.08	0.36	(111.88,127.47)	0.92	4.42	1.07
	n	194	194	180	185	184	194
C/T	Mean	15.1	90.3	119.35	28.5	244.1	60.1
	SE	0.11	0.55	(107.69,132.27)	1.32	6.34	1.65
	n	76	76	73	71	71	77
T/T	Mean	15.1	90.9	174.78	38.8	185.3	60.6
	SE	0.48	1.73	(103.56,294.97)	7.20	25.80	6.39
	n	5	5	4	4	4	5

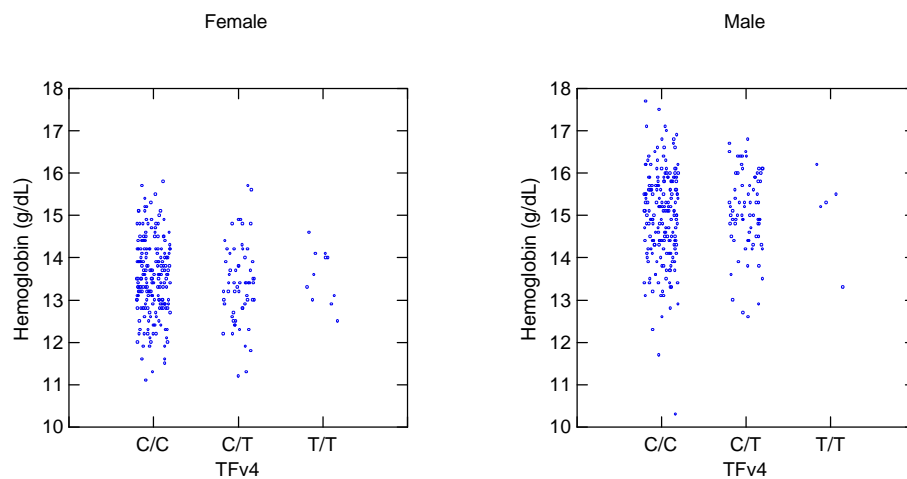
*Note.* \* Ferritin values are based on the logarithmic distribution. \*\* 95% confidence interval of the logarithmic distribution. SE, standard error of the mean.



**Figure 1.** The effect of the TFv4 genotype on the percent transferrin saturation of subjects homozygous for the *HFE* wild type.



**Figure 2.** The effect of the TFv4 genotype on the mean corpuscular volume of the red cells of subjects homozygous for the *HFE* wild type.



**Figure 3.** The effect of the TFv4 genotype on the hemoglobin level of subjects homozygous for the *HFE* wild type.

with each oligomer separately. After hybridizing for 16–24 hr, the membranes were washed for 20–25 min at 48 C for TFv1, TFv4 and TFv5, and at 47 C for TFv2 and TFv3. The filters were exposed to x-ray film and autoradiography performed.

## RESULTS AND DISCUSSION

We have genotyped 919 subjects undergoing health screening and 113 patients with documented hemochromatosis for five transferrin variants and for *HFE* mutations. The frequency of the TFv4 genotypes in 915 subjects of various ethnic backgrounds is presented in Table 4. The prevalence of TFv4 as identified by DNA analysis is consistent

with population studies that were performed using electrophoretic methods (8,11).

We examined the effect of the TFv4 polymorphism on the hemoglobin level, mean corpuscular volume (MCV), transferrin saturation (%TS) and unsaturated iron binding capacity (UIBC) in these 576 samples with the wild type *HFE* genotype (Table 5, Figures 1–3). We found that subjects who were heterozygous (C/T) or homozygous (T/T) for TFv4 exhibited normal levels of ferritin, transferrin saturation, unbound iron binding capacity, hemoglobin levels and MCV values when compared to samples with a normal with known *HFE* mutations at nt 187 or nt 845 also

transferrin genotype (C/C). Analysis of 343 subjects failed to show any effect of the TFv4 polymorphism on these parameters (Table 6).

The other transferrin variants are much less prevalent in the population and only a relatively few individuals were detected who were heterozygous for these mutations. Out of 919 samples examined,

**Table 6.** Effect of TFv4 on the Hemoglobin, Mean Corpuscular Volume (MCV) Transferrin Saturation (%TS), and Unsaturated Iron Binding Capacity (UIBC) of Individuals with at Least One *HFE* Mutant Allele

TFv4		Hemoglobin	MCV	Ferritin*	% TS	UIBC	Age
Female							
C/C	Mean	13.5	89.5	52.80	24.4	280.2	55.5
	SE	0.06	0.34	(49.48,56.33)**	0.80	4.82	0.97
	n	202	202	188	198	198	209
C/T	Mean	13.4	90.0	48.35	23.0	282.4	57.2
	SE	0.12	0.46	(43.51,53.72)	1.22	7.03	1.77
	n	61	61		59	59	61
T/T	Mean	13.6	88.2	37.43	17.7	288.7	58.3
	SE	0.20	0.93	(29.03,48.25)	2.02	13.42	4.87
	n	11	11	11	11	11	11
Male							
C/C	Mean	15.0	90.5	119.42	29.6	240.1	56.5
	SE	0.08	0.36	(111.88,127.47)	0.92	4.42	1.07
	n	194	194	180	185	184	194
C/T	Mean	15.1	90.3	119.35	28.5	244.1	60.1
	SE	0.11	0.55	(107.69,132.27)	1.32	6.34	1.65
	n	76	76	73	71	71	77
T/T	Mean	15.1	90.9	174.78	38.8	185.3	60.6
	SE	0.48	1.73	(103.56,294.97)	7.20	25.80	6.39
	n	5	5	4	4	4	5

Note. \* Ferritin values are based on the logarithmic distribution. \*\* 95% confidence interval of the logarithmic distribution. SE, standard error of the mean.

**Table 7.** Effect of Transferrin Mutations on the Hemoglobin, Mean Corpuscular Volume (MCV), Transferrin Saturation (%TS), and Unsaturated Iron Binding Capacity (UIBC) of Individuals with TFv1, TFv2, and TFv3 Mutations

	TFv1	TFv2	TFv3	TFv4	TFv5	<i>HFE</i>	Hb	MCV	Ferritin	% TS <sup>#</sup>	UIBC <sup>#</sup>	Sex	Age	Ethnic origin
Case 1	A/G	A/A	G/G	C/C	A/A	wt/wt*	14.6	95.6	38	62	118	M	42	Caucasian
Case 2	A/G	A/A	G/G	C/T	A/A	wt/187G	13.4	87.8	87	17	260	F	77	Caucasian
Case 3	A/G	A/A	G/G	C/C	A/A	wt/wt	12.2	84.5	62	15	263	F	48	African
Case 4	A/G	A/A	G/G	C/C	A/A	wt/wt	13.5	86	124	21	254	M	28	African
Case 5	A/A	A/G	G/G	C/C	A/A	wt/wt	13.7	90.7	210	20	279	F	70	Pacific Islander
Case 6	A/A	A/G	G/G	C/T	A/A	wt/wt	14.4	91	95	27	260	F	58	Asian
Case 7	A/A	A/G	G/G	C/C	A/A	845A/845A	15.5	96.3	872	73	77	M	46	Caucasian
Case 8	A/A	A/A	A/G	C/C	A/A	wt/wt	13.4	89.4	101	16	262	F	58	Caucasian
Case 9	A/A	A/A	A/G	C/T	A/A	wt/187G	15	95.4	76	35	186	M	78	Caucasian
Case 10	A/A	A/A	A/G	C/C	A/A	845A/wt	13.4	89.9	251	34	157	F	67	Caucasian
Case 11	A/A	A/A	A/G	C/C	A/A	wt/wt	12.9	91.7	35	13	326	F	52	Caucasian
Case 12	A/A	A/A	A/G	C/C	A/A	wt/187G	13.3	92.9	11	14	368	F	50	Caucasian

Note. \* = wild type

**Table 8.** Frequency of Transferrin Variants in 113 Patients with Clinical Hemochromatosis

HFE nt 845	TFv1		TFv2		TFv3		TFv4		TFv5	
	A	G	A	G	G	A	C	T	A	G
A/A (80)	1.0000	0.0000	0.9938	0.0062	0.9867	0.0133	0.8608	0.1392	1.000	0.000
A/G (7)	1.0000	0.0000	1.0000	0.0000	0.9286	0.0714	0.9286	0.0714	1.000	0.000
G/G (26)	1.0000	0.0000	1.0000	0.0000	1.0000	0.0000	0.9038	0.0962	1.000	0.000
<b>TOTAL</b> (113)	1.0000	0.0000	0.9956	0.0044	0.9860	0.0140	0.8750	0.1250	1.000	0.000

we found 4 heterozygous for TFv1, 3 heterozygous for TFv2 and 5 heterozygous for TFv3. No samples were found to have TFv5. The allele frequencies of TFv1, TFv2 and TFv3 were 0.0022, 0.0016, and 0.0027, respectively. Subjects with these transferrin variants exhibited blood iron parameters within the normal range when compared to the mean calculated and for their sex and *HFE* genotype. These results are shown in Table 7.

The penetrance of the homozygous *HFE* genotype seems to be relatively low with respect to clinical disease. Therefore, one would anticipate that if a mutation increased the severity of iron loading in patients who inherit this genotype it would be over-represented in the patient population when compared with the general population. This did not appear to be the case. As shown in Table 8, the gene frequency of the TFv4 T haplotype was 0.1392, a value very similar to the 0.1501 that we found in the general population (Table 4). Similarly, the frequency of this haplotype was not significantly altered in patients with hemochromatosis who did not have the homozygous 845A genotype. Similarly, the frequency of the less common TFv1, TFv2, TFv3, and TFv5 polymorphisms did not vary from that found in the general population.

Our studies indicate that the polymorphic transferrin variants investigated do not have a significant effect on iron metabolism and cannot explain the remarkable phenotypic variability that exists in hereditary hemochromatosis. It is possible that other mutations in transferrin might be responsible for some of the variability; indeed, patients with atransferrinemia are iron-loaded. However, it is likely that there are other gene(s) that play a more important role in modulating the effect of *HFE* mutations and in causing non-*HFE*-linked hemochromatosis.

## ACKNOWLEDGMENTS

This is manuscript 12907MEM from the Scripps Research Institute. This work was supported, in part, by Grants DK53505-02 (supplemented with a grant from the Division of Nutrition and Physical Activity, Centers for Disease Control and Prevention) and RR00833 from the NIH and by funds from the Stein Endowment. We greatly appreciate the assistance of the staff of the Kaiser-Permanente Department of Preventive Medicine in San Diego in making these studies possible.

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