REPORT

Variants in *TF* and *HFE* Explain ~40% of Genetic Variation in Serum-Transferrin Levels

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Only a small proportion of genetic variation in complex traits has been explained by SNPs from genome-wide association studies (GWASs). We report the results from two GWASs for serum markers of iron status (serum iron, serum transferrin, transferrin saturation with iron, and serum ferritin), which are important in iron overload (e.g., hemochromatosis) and deficiency (e.g., anemia) conditions. We performed two GWASs on samples of Australians of European descent. In the first GWAS, 411 adolescent twins and their siblings were genotyped with 100K SNPs. rs1830084, 10.8 kb 3′ of *TF*, was significantly associated with serum transferrin (p total association test = 1.0×10^{-9}; p within-family test = 2.2×10^{-5}). In the second GWAS on an independent sample of 459 female monozygotic (MZ) twin pairs genotyped with 300K SNPs, we found rs3811647 (within intron 11 of *TF*, HapMap CEU r^2 with rs1830084 = 0.86) was significantly associated with serum transferrin (p = 3.0×10^{-15}). In the second GWAS, we found two additional and independent SNPs on *TF* (rs1799852 and rs2280673) and confirmed the known C282Y mutation in *HFE* to be independently associated with serum transferrin. The three variants in *TF* (rs3811647, rs1799852 and rs2280673) plus the *HFE* C282Y mutation explained ~40% of genetic variation in serum transferrin (p = 7.8×10^{-25}). These findings are potentially important for our understanding of iron metabolism and of regulation of hepatic protein secretion, and also strongly support the hypothesis that the genetic architecture of some endophenotypes may be simpler than that of disease.

Iron is essential for biochemical functions such as oxygen transport and oxidative phosphorylation. Excessive iron can cause iron-overload-related liver diseases (e.g., hemochromatosis [MIM 235200]), whereas iron deficiency can lead to anemia. Iron status can be assessed by measuring the levels of serum iron, serum transferrin, transferrin saturation with iron, and serum ferritin. Approximately a quarter to a half of the variation in these iron markers is due to genetic factors.[1,2] The C282Y (a cystine to tyrosine mutation at position 282) and H63D (a histidine to aspartate mutation at position 63) mutations in the hemochromatosis gene (*HFE* [MIM 235200]) are known for affecting iron metabolism. These mutations can lead to genetic hemochromatosis, but they only explain a small proportion of the heritability of iron status (~5%).[1,2]

To find additional genetic variants associated with variation in iron status, we first conducted a GWAS on 411 individuals (twins and their sibs) from 150 Australian nuclear families of European descent using the Affymetrix Gene-Chip Human Mapping 100K Set, consisting of 50K XbaI and 50K Hind chips (100K GWAS). Subjects were recruited by the Genetic Epidemiology Laboratory of the Queensland Institute of Medical Research, Australia, as part of adolescent-twin studies on various phenotypes, including melanoma risk factors[3] and cognition.[4] We determined iron status by measuring the levels of serum iron (μmol/L), serum transferrin (g/L), and transferrin saturation with iron (%) and serum ferritin (μg/L) at up to three visits

(age 12, 14, and 16). Because of the skewed distribution, serum-ferritin levels were transformed ($\log_{10}$) before the analyses. Methods for phenotypic measurements have been described elsewhere.[2] To remove the need to accommodate the correlated nature of the data, we used the mean of the measurements across ages in the association analyses (Table 1 and Table S1 available online). The analysis on the mean of multiple measures is also more powerful than analysis that is based on a single measurement and discarding the others.

Initially, a total of 676 individuals (215 parents and 461 children) from 169 nuclear families were genotyped. Monozygotic (MZ) twin pairs were present in 64 families (58 MZ pairs, 4 MZ triplets, and 2 set of triplets containing an MZ pair). The number of children per family ranged from two to five with an average of 2.7. Both parents were genotyped in 104 families, one parent was genotyped in seven families, and no parent was genotyped for the remaining 58 families, but none of them had iron-related phenotypes measured. The expected relationships between members of a family were confirmed through the use of identity by state (IBS) statistics across all SNPs.

A total of 116,204 SNPs from the Affymetrix GeneChip Human Mapping 100K Set were genotyped in the sample. Nonautosomal SNPs were removed. Any discordant genotypes between MZ pairs were also removed. Parent-offspring genotype consistency was only checked for the XbaI chip because parents were only genotyped with that

Table 1. Descriptive Statistics of the Initial Sample from the 100K GWAS

Measure	Sex	N	Min	Max	Mean	SD
Age (years)	Male	203	10.05	18.34	14.28	1.6
	Female	208	10.22	19.19	14.19	1.77
	Total	411	10.05	19.19	14.23	1.68
Serum iron (µmol/L)	Male	203	6.65	36	17.54	4.90
	Female	206	4.3	33.8	16.99	5.29
	Total	409	4.3	36	17.26	5.11
Serum transferrin (g/L)	Male	203	2.25	3.73	2.93	0.33
	Female	208	2.01	4.15	2.94	0.37
	Total	411	2.01	4.15	2.93	0.35
Transferrin saturation (%)	Male	203	8.48	47.64	24.22	6.95
	Female	206	5.6	57.34	23.45	7.60
	Total	409	5.6	57.34	23.83	7.29
Log_{10} (serum ferritin) µg/L	Male	203	0.76	2.27	1.67	0.21
	Female	208	0.7	2.21	1.56	0.26
	Total	411	0.7	2.27	1.61	0.24

Note that the values are the mean of measurements at up to three visits.

Table 2. Descriptive Statistics of the Second GWAS Sample from the 300K GWAS

Measure	Sex	N (pairs)	Min	Max	Mean	SD
Age (years)	Female	441	30.00	91.00	46.71	12.27
Serum iron (µmol/L)	Female	441	4.35	43.70	17.26	5.85
Serum transferrin (g/L)	Female	441	1.53	5.30	2.92	0.50
Transferrin saturation (%)	Female	439	5.30	72.90	24.15	8.72
Log_{10} (serum ferritin) µg/L	Female	441	0.70	3.05	1.81	0.38

Note that the values are the mean of measurements of the MZ pairs.

chip. Genotypes for all family members were removed in the case of a mismatch. After these removals, there were 113,199 SNPs. From these SNPs, we further removed 3,876 monomorphic SNPs, 1,884 SNPs that failed the Hardy-Weinberg Equilibrium (HWE) test (using the Mendel Statistical Package[5]) at $p \leq 0.001$, 6,592 SNPs in which the minor allele frequency (MAF) was less than 0.01, and one duplicate SNP. After the cleaning process, there were 100,846 SNPs that were tested for their association with the phenotypes related to iron status.

We removed 19 families that were ethnic outliers on the basis of either self-reported ancestry or a multidimensional scaling (MDS) plot (Figure S1). To avoid disproportionate contribution to the test statistics from a very small number of individuals with extreme phenotypes, we also removed individuals where their residuals after correcting for covariates (sex, age, age^2, sex*age, and $sex*age^2$) were more than ± 4 SD from the mean (i.e., one individual for transferrin saturation, two individuals for serum ferritin, and none for serum iron and serum transferrin) for any given phenotype. The final data consisted of 411 phenotyped individuals (twins and their siblings) from 150 nuclear families. The means of the standardized residuals after covariate correction were used for the analyses.

Family-based genome-wide association analyses were performed in QTDT,[6] where both total and within family association tests were performed with an additive model. Both tests use variance components to model the phenotypic similarities between related individuals. Although the within-family association test is robust to population stratification, its power is limited compared to the total association test because it only uses information from individuals with heterozygous parents. The model included an additive genetic effect for the SNP, a polygenic effect, an environmental effect common to all family members, an environmental effect common to twins, and a residual individual-specific environmental effect.

The second GWAS was performed in an independent sample of 459 female monozygotic (MZ) twin pairs, genotyped with 315,887 SNPs from the Illumina Human-Hap300 (300K GWAS). The subjects were Australian adult female MZ twin pairs of European descent recruited through the Australian Twin Registry. They were part of a GenomEUtwin study[7] that used genome-wide analyses of twin and population cohorts to identify genes in common diseases. They had a mean of age of 46.71 years and the descriptive statistics of the final data set are presented in Table 2 and Table S2. The measurements of iron status have been described elsewhere.[2]

Initially, there were 477 individuals (one individual from each MZ pair) genotyped with the Illumina Human-Hap300 (300K GWAS). We removed 15 individuals because of low genotyping rate (≤ 0.90). From the MDS plot (Figure S2), three individuals were identified to be of mixed ancestry and thus removed from the analyses. As with the 100K GWAS, for any given phenotype we also removed individuals in which their residuals after correcting for covariates (i.e., age) were more than ± 4 SD from the mean (i.e., three, two, and five individuals for serum iron, serum transferrin, and transferrin saturation, respectively, and none for serum ferritin). There were 318,237 SNPs genotyped in the 300K GWAS sample. As part of the QC process, we removed 548 SNPs that failed the HWE test ($p \leq 0.001$), 1,605 SNPs with more than 10% missing genotypes, and 291 SNPs with MAF < 0.01. Thus, the final number of twin pairs and SNPs used in the association analyses were 459 and 315,887, respectively.

The MZ phenotypes were first adjusted for the effect of age (i.e., -0.063 µmol/L/year, -0.007 g/L/year, -0.045%/year, and 0.012/year for serum iron, serum transferrin, transferrin saturation, and serum ferritin, respectively). The mean of the standardized residuals for the two members of each MZ pair was used for the analyses. The association was analyzed in PLINK with an additive genetic model.[8] Visualization and annotation of the GWAS results were performed in WGAViewer.[9]

From the first (adolescent) GWAS, SNP rs1830084 (MAF 0.36), 10.8 kb $3'$ of the transferrin gene (*TF* [MIM 190000]), was the most significantly associated SNP with serum transferrin (p total association test = 1.0×10^{-9}; p within-family test = 2.2×10^{-5}) (Table S3). Each copy of the *A* allele of rs1830084 increased serum-transferrin levels by 0.43 SD of the mean phenotype, or ~0.38 phenotypic
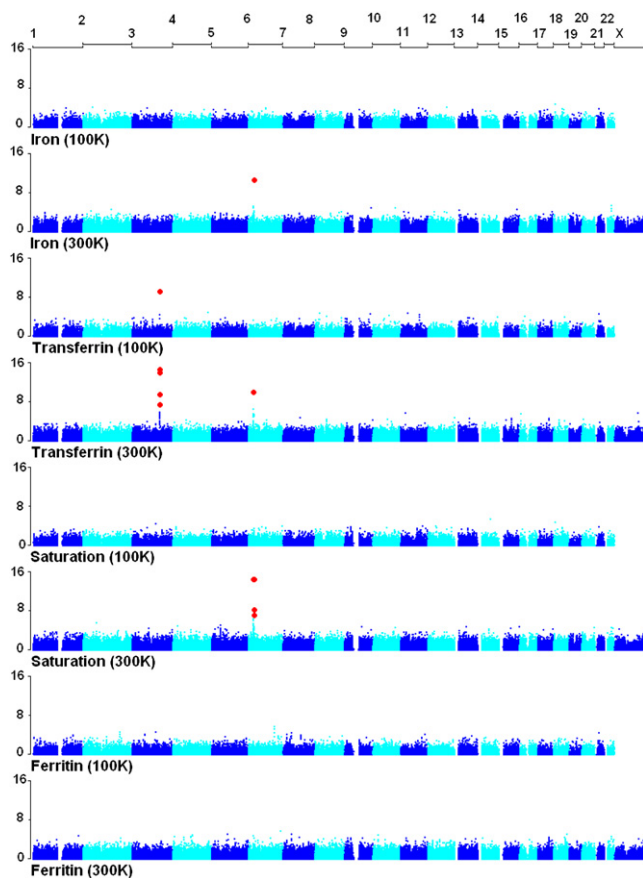
Figure 1. GWAS Results for Iron Status in the 100K and 300K Studies

On the x and y axis are chromosome number and $-\log_{10}(p)$ value, respectively. Red dots indicate the SNPs that reached genome-wide significance ($p < 0.05$) after a Bonferroni correction for multiple testing.

SD. The effect was not significantly different between males and females ($p = 0.23$). This SNP explained ~7.7% of variation in serum transferrin and had no effect on the other iron markers. There were no typed SNPs significantly associated with other iron status phenotypes (Figure 1).

SNP rs1830084 (which had shown significant results in the first GWAS) was not on the HumanHap300 chip used for the second GWAS, but two SNPs in high-linkage disequilibrium (LD) with it were, rs3811647 (within intron 11 of *TF*, HapMap CEU $r^2 = 0.86$) and rs6794945 (20.8 kb from 3' of *TF*, HapMap CEU $r^2 = 0.93$), and these SNPs were significantly associated with serum-transferrin concentration ($p = 3.0 \times 10^{-15}$ and 5.0×10^{-15}, respectively) (Figures 1 and 2). These were the top two SNPs based on their p values (Table S4), and their effects were inseparable because of their high LD ($r^2 = 0.86$). rs3811647 was also associated with transferrin saturation ($p = 0.002$) and serum ferritin ($p = 0.03$), but not with serum iron ($p = 0.81$). The effect of rs3811647 on serum transferrin was additive with each *A* allele increasing serum transferrin by 0.46 SD of the MZ mean, or ~0.40 phenotypic SD. This SNP explains 13% of the variation in MZ means or ~10.3% of serum-transfer-

rin variation in the population. Its effect on the other iron phenotypes was much smaller (2.1 and 1.1% of the variation in MZ means for transferrin saturation and serum ferritin) (Table 3).

In addition to SNPs in *TF*, we also confirmed the association[2] of the known C282Y mutation in *HFE* (rs1800562) with iron status in the 300K GWAS. rs1800562 was associated with serum iron ($p = 3.5 \times 10^{-11}$), serum transferrin ($p = 1.1 \times 10^{-10}$), transferrin saturation ($p = 4.3 \times 10^{-15}$), and serum ferritin ($p = 4.5 \times 10^{-5}$). rs1800562 explained 9.5%, 9.1%, 13.2%, and 3.7% of the variation in MZ means of serum-iron, serum-transferrin, transferrin-saturation, and serum-ferritin levels, respectively (Table 3).

The C282Y mutation (rs1800562) was not part of the 100K Affymetrix GeneChip, and there was no proxy for it. The other *HFE* mutation, i.e., H63D mutation (rs1799945) was neither on 100K nor 300K chips, and there was no proxy for it. Therefore, we could not determine the effect of either C282Y or H63D mutations in the 100K GWAS and could not redetermine the contribution of the H63D mutation to the variation in iron status in the 300K GWAS.

Recently, Finberg et al.[10] demonstrated that iron-deficiency anemia refractory to oral iron therapy can be caused by mutations in the transmembrane protease, serine 6 gene (*TMPRSS6* [MIM 609862]). This gene codes for a transmembrane serine protease, which has a role in detecting iron deficiency and regulating hepcidin expression.[11] In our 300K GWAS, several SNPs within this gene were associated with serum-iron levels and transferrin saturation (Table S5); in particular, a synonymous coding SNP in exon 13 (rs4820268, $p = 3.7 \times 10^{-6}$ and 2.0×10^{-5}, respectively).

In the 300K GWAS, there were 16 additional SNPs within 60 kb of *TF* associated with serum transferrin at nominal $p < 0.05$ (Table S6). To determine whether there are multiple SNPs in this region contributing to variation in serum transferrin, we fitted a stepwise multiple regression including those SNPs (excluding rs6794945, which is highly correlated with rs3811647 [HapMap CEU $r^2 = 0.86$]) together with the *HFE* C282Y mutation. As a result, we found two additional SNPs (in addition to rs3811647), rs1799852 (MAF 0.09), and rs2280673 (MAF 0.34), which were independently influencing variation in serum transferrin (nominal $p = 4.7 \times 10^{-6}$ and 2.3×10^{-4}, respectively). These SNPs explained an additional 2.2% and 1.7% of variation in MZ mean of serum transferrin. These estimates are likely to be biased upward by multiple testing, but the ascertainment (2 out of 16) was not strong and significance was high, so the bias is likely to be small. rs1799852 and rs2280673 were also associated with serum ferritin ($p = 0.04$ and 0.003) but not with serum iron or transferrin saturation.

Genetic variants within *TF* have previously been studied for their association with iron status,[12–16] but to our knowledge the associations between SNPs rs3811647, rs1799852, and rs2280673 with serum transferrin levels have not been reported elsewhere. In addition, the main *TF* SNP, rs3811647, is not only common in our samples
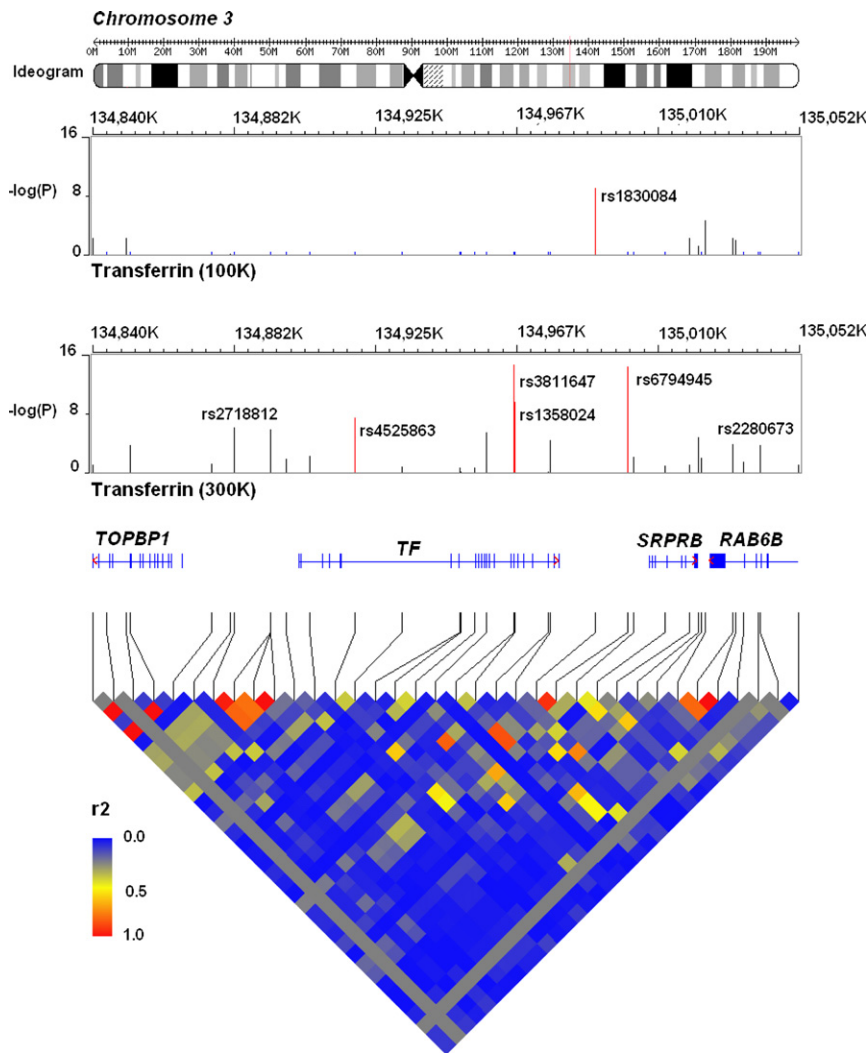
Figure 2. The Plots of $-\log10(P)$ for the Associations between SNPs in TF $\pm$ 60 kb and Serum-Transferrin Levels in 100K and 300K GWAS

The linkage disequilibrium (LD) plot for the SNPs in the region from the HapMap CEU population is shown in r^2.

family test of the association removed the effect of population stratification because it tested the alleles that were transmitted from the parents to their offspring. In addition, lambda values[18] of 1.001, 0.999, 1.009, and 1.003 for the total association tests of serum iron, serum transferrin, transferrin saturation, and serum ferritin as well as Q-Q plots indicated that the residual population-stratification effects were very small or absent (Figure S3). The corresponding lambda values for the 300K GWAS of 1.004, 1.003, 1.015, and 1.001 and Q-Q plots also indicated that the effect of population stratification on this sample was minimal (Figure S4).

Both GWAS analyses were performed on the mean of a number of observations. In the 100K GWAS, the mean was over repeated observations per individual, varying from one to three (Table 1 and Table S1). In the 300K GWAS, the mean was from observations on pairs of monozygotic twins (Table 2 and Table S2). In general, the variance of the mean of *n* observations is $[(1 - r)/n + r]\mathrm{var}(y)$, with *r* representing the intraclass correlation and var(*y*) representing the variance in the population. For obtaining the estimated effect size and the proportion of variance explained in the population, the estimates from the means should therefore be

but also in other human populations. A MAF of 0.36, 0.36, 0.52, and 0.23 were reported in the four HapMap populations (CEU, CHB, JPT, and YRI, respectively). The C282Y mutation is only polymorphic in the CEU population with a MAF of 0.04.[17]

The reported associations in these studies were robust to population stratification. In the 100K GWAS, the within-

Table 3. Additive Effects $\pm$ SE of Significant SNPs on Iron Status in Female MZ Twins in the 300K GWAS

Gene	SNP	Serum Iron			Serum Transferrin			Transferrin Saturation			Serum Ferritin		
		Effect in SD	R^2	p	Effect in SD	R^2	p	Effect in SD	R^2	p	Effect in SD	R^2	p
TF	rs3811647	-0.01 ± 0.06	0.00	0.81	0.46 ± 0.06	0.13	3.0×10^{-15}	-0.17 ± 0.06	0.02	2.1×10^{-3}	-0.13 ± 0.06	0.01	0.03
TF	rs1799852	0.03 ± 0.09	0.00	0.71	-0.43 ± 0.09	0.05	4.7×10^{-6}	0.17 ± 0.09	0.01	0.06	0.20 ± 0.10	0.01	0.04
TF	rs2280673	0.01 ± 0.06	0.00	0.84	0.22 ± 0.06	0.03	2.3×10^{-4}	-0.04 ± 0.05	0.00	0.42	-0.18 ± 0.06	0.02	2.5×10^{-3}
HFE	rs1800562	0.66 ± 0.10	0.10	3.5×10^{-11}	-0.68 ± 0.10	0.09	1.1×10^{-10}	0.80 ± 0.10	0.13	4.3×10^{-15}	$0.44 \pm .11$	0.04	4.5×10^{-5}
TMPRSS6	rs4820268	-0.24 ± 0.05	0.05	3.7×10^{-6}	-0.01 ± 0.06	0.00	0.87	-0.22 ± 0.05	0.04	1.96×10^{-5}	0.01 ± 0.06	0.00	0.80

R^2 is the proportion of phenotypic variance in MZ means explained by the SNP.

scaled with the ratio of the standard deviations of the mean phenotype and individual phenotypes (Tables S1 and S2). Similarly, the proportion of phenotypic variance explained in the population is $[(1 - r)/n + r]$ multiplied by the proportion of variance of the mean phenotypes explained by a SNP. For example, the mean of serum transferrin in 300K GWAS was based on $n = 2$ and r ~0.56, so that the variance of the means is ~0.78 of the variance in the population. Therefore, the proportion of phenotypic variance explained in the population is 0.78 of the proportion of variance in the mean phenotype. The effect size in phenotypic standard deviations is $\sqrt{0.78}$ ~0.88 of the effect size expressed in standard deviations of the mean phenotype.

From a database of gene expression,[19] we found that two SNPs that significantly affected serum-transferrin concentration within *TF* in the 300K GWAS (i.e., rs1358024 and rs1115219, HapMap CEU r^2 with rs3811647 is 0.38 and 0.18, respectively) were also significantly associated with the level of mRNA expression of the signal-recognition particle receptor, B subunit gene (*SRPRB*) (p = 3.5×10^{-10} and 1.9×10^{-8}, respectively). This gene is 27 kb distant from *TF* and encodes the signal-recognition particle receptor that is required for targeting of secreted proteins[20] (e.g., serum transferrin). We also identified a SNP in *SRPRB* (rs10512913, HapMap CEU r^2 with rs3811647 is 0.18) that was associated with both our serum-transferrin concentration and the expression of *SRPRB* mRNA (p = 2.0×10^{-5} and 2.1×10^{-8}, respectively) (Tables S6 and S7). These observations are consistent with a possible causative relationship between *SRPRB*-transcript variation and serum-transferrin concentration.

The presented statistical significances and the estimated effect sizes should be viewed in terms of the complexity of the present study. First, all presented p values were unadjusted for multiple comparison. For the 100K GWAS, the reference p values after Bonferroni correction that correspond to global significance at 5% and 1% were 5×10^{-7} and 10^{-7}, respectively. The corresponding p values for the 300K GWAS were 1.6×10^{-7} and 3.2×10^{-8}, respectively. Second, the estimated proportions of genetic variance explained by SNPs were based on the assumption that the estimated proportion of phenotypic variance and heritability were accurate.

The samples used in this study were adolescent twins and their siblings (the 100K GWAS) and adult female MZ twins (the 300K GWAS). There were a number of issues that were related to these samples and that are worth discussion. First, the generalization of the results from samples of twins may not be applicable to the general population. However, to our knowledge there is no evidence to suggest that there are phenotypic differences in the relevant age groups between twins and nontwins in relation to serum markers of iron status. Second, the subjects who participated in this study were volunteers and may not be a random sample from the population. However, it is difficult to see how this type of participation bias will have effects on the association between SNPs and phenotypes such as serum

markers of iron status. Third, the association between the genetic variant in *TF* (rs1830084 or rs3811647) and serum transferrin was evident in two samples with different age ranges. This indicated that the SNPs were associated with serum transferrin in both adolescent and adult samples.

Variations in serum markers for iron status are known to be influenced by the time of the day when the blood was collected (particularly for serum iron)[2] and menopausal status (particularly for serum ferritin).[21] In the 100K GWAS, the subjects were adolescents and their bloods were collected at approximately the same time of the day for all subjects. However, in the 300K GWAS, the bloods were collected differently throughout the day and some subjects had reached menopause. To check whether the effects of SNPs on the serum markers of iron status were confounded by the effect of the collection time and menopausal status, we performed additional association analyses in the 300K GWAS by adjusting for the effects of the collection time and menopausal status. The correlations between $-\log_{10}(p)$ of the association tests by including and excluding the collection time and menopausal status as covariates were very high (range: 0.89–0.99) (Figure S5). Importantly, this adjustment did not have any effect on the top hits (top right-hand corners in Figure S5). Hence, the results presented were not confounded by the effects of the collection time and menopausal status.

Overall, the genetic variants identified in 300K GWAS (three SNPs in *TF* [rs3811647, rs1799852, and rs2280673] and the C282Y mutation in *HFE*) explained 26% of the serum-transferrin level variation between MZ means, or ~20% of the phenotypic variance. Whitfield et al.[2] estimated the heritability of serum transferrin for women to be 0.49. Therefore, ~40% of the genetic variation in serum transferrin in the population can be explained by only a handful of genetic variants in two genes. This finding is remarkably different from that reported for other disease and quantitative traits.[22,23] All significant *TF* SNPs had pleiotropic effects on serum ferritin, but only rs3811647 had a pleiotropic effect on transferrin saturation. The pleiotropic effects of the *TF* SNPs on serum iron were not statistically significant. In addition, we have replicated results showing that the C282Y mutation in *HFE* has a significant effect on iron status and provide evidence that common variation in *TMPRSS6* is associated with serum iron and transferrin saturation. Our results are important for understanding of iron metabolism and iron-related disorders, such as anemia and iron overload. Because a large proportion of genetic variation in serum-transferrin levels can be explained by only a handful of SNPs at two loci, this is an example of a relatively simple genetic architecture of an endophenotype. GWASs on such traits may be more efficient and effective than GWASs for disease.

Supplemental Data

Supplemental Data include seven tables and six figures and can be found with this article online at http://www.ajhg.org/.

Web Resources

The URL for data presented herein is as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/

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