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TMPRSS6, but not *TF*, *TFR2* or *BMP2* variants are associated with increased risk of iron-deficiency anemia

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A variety of conditions lead to anemia, which affects one-quarter of the world's population. Previous genome-wide association studies revealed a number of genetic polymorphisms significantly associated with plasma iron status. To evaluate the association of genetic variants in genes involved in iron delivery and hepcidin regulation pathways with the risk of iron-deficiency anemia (IDA), the following single nucleotide polymorphisms were genotyped in 2139 unrelated elderly Chinese women: rs3811647 (*TF*), rs7385804 (*TFR2*), rs235756 (*BMP2*), and rs855791(V736A) and rs4820268 (*TMPRSS6,* encoding matriptase-2). We identified common variants in *TMPRSS6* as being genetic risk factors for both iron deficiency ($OR_{rs855791} = 1.55$, $P = 4.96 \times 10^{-8}$) and IDA ($OR_{rs855791} = 1.78$, $P = 8.43 \times 10^{-9}$). *TMPRSS6* polymorphisms were also associated with lower serum iron (SI) and hemoglobin levels, consistent with their associations to increased iron deficiency and anemia risk. Variants rs3811647 in *TF* and rs7385804 in *TFR2* were associated with reduced SI, serum transferrin and transferrin saturation levels; however, these variants were not associated with iron deficiency or anemia risk. Our findings suggest that *TF*, *TFR2* and *TMPRSS6* polymorphisms are significantly associated with decreased iron status, but only variants in *TMPRSS6* are genetic risk factors for iron deficiency and IDA.

INTRODUCTION

Anemia is a worldwide blood disorder affecting about one-quarter of the world's population, especially pregnant women and young children due to their high iron requirements (1). A number of conditions are associated with anemia, including nutritional or absorptional deficiency, infectious diseases, blood loss caused by menstruation or parasitic diseases and genetic mutations (2). Iron-deficiency anemia (IDA) is the most common type of anemia and is caused by inadequate iron availability for hemoglobin production, due to the lack of dietary iron or insufficient uptake of iron. In all, about half of anemia cases result from iron deficiency (1). Besides nutritional factors and infectious diseases, genetic mutations have been linked to hereditary anemia. These include mutations in genes encoding hemoglobin subunits, leading to thalassemia (3), and mutations in the *TMPRSS6* gene, causing iron-refractory iron-deficiency anemia (IRIDA) (4).

Several indicators, such as hemoglobin, serum iron (SI) and serum ferritin, have been selected as clinical parameters that reflect the iron metabolism status of the body (2). In recent years, association and genome-wide association (GWA) studies of these parameters have revealed a number of

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genetic variants and furthered our understanding of iron metabolism. For example, polymorphisms in the human hemochromatosis (*HFE*) gene account for higher SI and transferrin saturation (TS) levels (5–7). The protein encoded by *HFE* is thought to be involved in the regulation of hepcidin (8,9), a 25-amino acid peptide regarded as the central regulator of iron metabolism (10). Similar to *HFE*, variants in genes involved in the signaling pathway controlling hepcidin production also showed associations with iron status parameters in humans (6,11,12). Among these genes, common variants in *TMPRSS6* were significantly associated with reduced SI (6,13), hemoglobin concentrations (14–16) and hepcidin levels (17), which were consistent with previous studies from *TMPRSS6* mutations in human (4) and mice (18,19).

Here, we performed a population-based analysis of elderly Chinese women with anemia from three geographic regions to assess the association of genetic variants in genes participating in iron delivery and hepcidin regulation with the risk of anemia. With reference to previous association studies, we selected single nucleotide polymorphisms (SNPs) in *TF*, *TFR2*, *BMP2* and *TMPRSS6* to evaluate their associations with iron-related traits, iron deficiency and anemia risk in Chinese women. In previous GWA studies, the SNP rs3811647 in *TF* was shown to be significantly associated with increased serum transferrin and total iron-binding capacity (TIBC) (6,7,14). The SNP rs7385804 in *TFR2* was found to be associated with SI and several other iron traits (12,16,20). In two association studies of iron overload disorders, the SNP rs235756 in *BMP2* showed an association with decreased serum ferritin level (11,21). Despite the prevalence of C282Y and H63D mutations of the *HFE* gene in Europeans, allelic frequencies of these two variants are rare in Chinese (data from HapMap). Therefore, variants in the *HFE* gene were excluded from our study.

This is the first time a population-based cohort study has been performed to determine the associations of genetic variants with the risk of iron deficiency and anemia. We found that a SNP in the *TF* gene, encoding the iron-binding protein transferrin, showed significant association with reduced serum transferrin and TIBC, as previously reported (6,7,14), but had no association with iron deficiency or anemia risk. In addition, a novel association between a polymorphism in the *TFR2* gene and TS was found. Notably, we show that while two common variants, rs855791 (V736A) and rs4820268, in the *TMPRSS6* gene were significantly associated with several iron traits and increased risk of anemia, polymorphisms in *TF*, *TFR2* and *BMP2* were not associated with an increased risk of anemia. Our results demonstrate that the *TMPRSS6* polymorphisms rs855791 and rs4820268 are genetic risk factors for iron deficiency and IDA.

RESULTS

Characteristics of study population

There were 2139 case–control samples from three places in China. Populations in our study were elderly women aged from 50 to 70 years old (Table 1). Therefore, differences in iron traits caused by sex were eliminated. Ages of people in case and control group were comparable. Women with anemia have lower hemoglobin, SI, serum ferritin and TS levels. Free erythrocyte protoporphyrin (FEP) levels and FEP/hemoglobin ratios were higher in anemic persons (Table 1). Genotype distributions in three subsets did not deviate from the Hardy–Weinberg equilibrium (Supplementary Material, Table S1).

Associations of genetic variants with iron traits and anemia risk

First, we tested associations between the selected genetic variants and common iron traits. Transferrin is the major iron delivery protein in the circulation, and polymorphisms in the *TF* gene have been reported to be associated with decreased serum transferrin levels (6) and TIBC (7). We confirmed the association of the SNP rs3811647 in *TF* with serum transferrin (Han $P = 2.56 \times 10^{-33}$, Zhuang $P = 4.86 \times 10^{-19}$) and TIBC in our study (Han $P = 5.99 \times 10^{-24}$, Zhuang $P = 4.21 \times 10^{-12}$) (Table 2). Although rs3811647 showed strong associations with serum transferrin and TIBC, no significant association with anemia risk was found (Table 3). The SNP rs7385804 in *TFR2* was found to be associated with SI (Han

Table 1. Characteristics of study population

	Han^{East}		Han^{North}		Zhuang	
	Anemia	Control	Anemia	Control	Anemia	Control
n	567	574	142	212	307	323
Age, year	62.46 ± 7.70	61.93 ± 7.69	58.12 ± 8.06	58.23 ± 7.03	60.30 ± 8.08	58.51 ± 7.68
Hemoglobin, g/l	112.63 ± 6.57	137.56 ± 7.30	113.36 ± 6.85	140.38 ± 9.74	111.38 ± 7.51	138.48 ± 7.20
Serum iron, μmol/l	13.75 ± 5.51	16.74 ± 5.91	14.17 ± 5.60	16.02 ± 5.07	13.11 ± 6.99	14.40 ± 5.54
Ferritin, ng/ml	119.28 ± 88.20	144.19 ± 90.16	92.68 ± 76.31	118.43 ± 86.02	139.87 ± 108.69	168.82 ± 116.75
Transferrin, mg/dl	259.29 ± 46.17	257.98 ± 35.58	259.99 ± 45.69	273.02 ± 43.99	242.93 ± 42.50	242.41 ± 32.52
TS, %	23.21 ± 9.51	27.55 ± 9.69	23.31 ± 10.13	25.47 ± 8.42	24.88 ± 13.30	25.26 ± 10.45
TIBC, μmol/l	60.29 ± 10.27	61.32 ± 9.44	63.10 ± 11.22	63.91 ± 10.06	55.15 ± 11.18	58.18 ± 8.48
FEP, μg/dl	40.03 ± 19.88	38.44 ± 17.36	49.04 ± 25.08	42.22 ± 15.33	27.44 ± 19.08	23.47 ± 11.46
FEP/Hb, μg/g	3.59 ± 1.91	2.80 ± 1.25	4.38 ± 2.38	3.02 ± 1.12	2.49 ± 2.18	1.70 ± 0.83

Data are mean ± SEM. Anemia was defined as hemoglobin concentration <120 g/l. Ferritin (ng/ml) indicates the serum ferritin level. Transferrin (mg/dl) indicates the serum transferrin level; TS, transferrin saturation; TIBC, total iron-binding capacity; FEP, free erythrocyte protoporphyrins; FEP/Hb, free erythrocyte protoporhyrins divided by the hemoglobin level.

Table 2. Associations of genetic variants with iron traits

SNP (risk allele)	Locus	Han^{East}			Han^{North}			Zhuang			Han^{a}			Han + $Zhuang^{b}$		
		Beta	SE	*P*-value	Beta	SE	*P*-value	Beta	SE	*P*-value	Beta	SE	*P*-value	Beta	SE	*P*-value
rs855791 (A)	*TMPRSS6*															
Hemoglobin		−2.31	0.59	1.01×10^{-4}	−2.65	1.20	0.03	−0.87	0.90	0.33	−2.38	0.53	3.57×10^{-6}[c]	−1.99	0.46	6.51×10^{-5}[c]
Serum iron		−1.41	0.24	6.90×10^{-9}	−1.45	0.40	3.70×10^{-4}	−1.23	0.36	7.76×10^{-4}	−1.42	0.21	2.84×10^{-11}[c]	−1.37	0.18	8.28×10^{-14}[c]
TS, %		−0.02	0.004	9.92×10^{-10}	−0.03	0.01	9.74×10^{-5}	−0.02	0.007	8.39×10^{-4}	−0.03	0.004	1.32×10^{-12}[c]	−0.02	0.003	4.47×10^{-15}[c]
FEP		2.08	0.78	0.007	0.66	1.46	0.65	0.65	0.92	0.48	1.76	0.69	0.01	1.36	0.55	0.013
FEP/HB		0.25	0.07	3.66×10^{-4}	0.11	0.13	0.43	0.04	0.10	0.70	0.22	0.06	0.002[c]	0.17	0.05	0.007[c]
rs4820268 (G)	*TMPRSS6*															
Hemoglobin		−2.01	0.59	6.61×10^{-4}	−3.37	1.20	0.005	−0.60	0.92	0.51	−2.27	0.53	8.67×10^{-5}[c]	−1.85	0.46	2.60×10^{-4}[c]
Serum iron		−1.20	0.24	8.31×10^{-7}	−1.41	0.40	5.45×10^{-4}	−1.38	0.37	2.23×10^{-4}	−1.25	0.21	7.52×10^{-9}[c]	−1.28	0.18	6.42×10^{-12}[c]
TS, %		−0.02	0.004	1.63×10^{-6}	−0.02	0.01	0.001	−0.03	0.01	7.66×10^{-5}	−0.02	0.004	3.43×10^{-8}[c]	−0.02	0.003	5.31×10^{-10}[c]
FEP		2.52	0.77	0.001	1.62	1.53	0.29	1.07	0.93	0.25	2.34	0.69	0.003	1.89	0.55	0.003[c]
FEP/HB		0.27	0.07	7.95×10^{-5}	0.21	0.14	0.14	0.07	0.10	0.49	0.26	0.06	1.32×10^{-4}[c]	0.21	0.05	3.97×10^{-4}[c]
rs3811647 (A)	*TF*															
Hemoglobin		−1.02	0.01	0.50	1.26	1.25	0.31	−0.29	0.83	0.73	−0.58	0.55	0.30	0.49	0.46	0.29
Serum iron		0.52	0.25	0.042	0.01	0.43	0.98	−0.76	0.34	0.03	0.38	0.22	0.08	0.05	0.18	0.7832
Transferrin		18.46	1.68	8.18×10^{-27}	16.42	3.36	1.53×10^{-6}	17.61	1.91	4.86×10^{-19}	18.05	1.50	1.28×10^{-32}[c]	17.88	1.18	3.77×10^{-51}[c]
TIBC		3.71	0.41	7.49×10^{-19}	3.56	0.79	8.57×10^{-6}	3.70	0.52	4.21×10^{-12}	3.67	0.36	3.00×10^{-23}[c]	3.68	0.30	3.40×10^{-34}[c]
rs7385804 (C)	*TFR2*															
Hemoglobin		0.007	0.76	0.99	2.62	1.46	0.07	−0.51	0.89	0.56	0.56	0.67	0.40	−0.009	0.59	0.99
Serum iron		−0.90	0.31	0.004	−1.52	0.49	0.002	−1.20	0.51	0.02	−1.08	0.26	2.34×10^{-6}[c]	−1.10	0.23	1.17×10^{-5}[c]
TS, %		−0.02	0.01	4.15×10^{-4}	−0.02	0.01	0.003	0.01	0.01	0.35	−0.02	0.004	2.23×10^{-5}[c]	−0.01	0.004	3.92×10^{-3}[c]
FEP		0.31	0.99	0.76	4.35	1.83	0.02	−2.89	1.28	0.02	1.23	0.87	0.16	−0.08	0.72	0.91

An additive genetic model was assumed and all regressions were adjusted for age. Ferritin (ng/ml) indicates the serum ferritin level. Transferrin (mg/dl) indicates serum transferrin level; TS, transferrin saturation; TIBC, total iron-binding capacity; FEP, free erythrocyte protoporphyrins; FEP/Hb, free erythrocyte protoporhyrins divided by the hemoglobin level. rs235756 in *BMP2* had no association with any traits in test, which was not included in the table. Beta, effect size of risk allele; SE, the standard error of beta coefficient.

[a]Combined results using meta-analysis of Han^{East} and Han^{North}.

[b]Combined results using meta-analysis of Han^{East}, Han^{North} and Zhuang.

[c]*P*-value after Bonferroni correction.

Table 3. Associations of genetic variants with the anemia risk

SNP (risk allele)	Gene	Han^{East}	Han^{North}	Zhuang	Han[a]	Han + Zhuang[b]
rs855791 (A)	*TMPRSS6*					
OR		1.39	1.47	1.05	1.41	1.29
95% CI		1.18–1.64	1.07–2.00	0.83–1.32	1.21–1.63	1.14–1.46
P-value		1.02×10^{-4}	0.02	0.71	2.57×10^{-5}[c]	2.47×10^{-4}[c]
rs4820268 (G)	*TMPRSS6*					
OR		1.29	1.58	1.06	1.35	1.26
95% CI		1.10–1.52	1.15–2.16	0.84–1.35	1.17–1.56	1.12–1.43
P-value		0.002	0.005	0.62	2.81×10^{-4}[c]	1.11×10^{-3}[c]
rs3811647 (A)	*TF*					
OR		1.09	0.93	1.05	1.05	1.05
95% CI		0.92–1.29	0.68–1.29	0.85–1.30	0.91–1.22	0.93–1.19
P-value		0.32	0.674	0.65	0.49	0.41
Rs7385804 (C)	*TFR2*					
OR		0.96	0.82	1.04	0.93	0.97
95% CI		0.78–1.19	0.57–1.19	0.83–1.31	0.78–1.11	0.84–1.12
P-value		0.73	0.30	0.72	0.42	0.68
rs235756 (G)	*BMP2*					
OR		0.90	0.83	1.18	0.88	0.96
95% CI		0.72–1.13	0.55–1.27	0.85–1.63	0.72–1.08	0.81–1.13
P-value		0.36	0.39	0.32	0.22	0.60

An additive genetic model was assumed and regressions were adjusted for age.
[a]Combined results using meta-analysis of Han^{East} and Han^{North}.
[b]Combined results using meta-analysis of Han^{East}, Han^{North} and Zhuang.
[c]*P*-value after Bonferroni correction.

$P = 4.15 \times 10^{-5}$, Zhuang $P = 0.0196$) and TS (Han $P = 4.45 \times 10^{-6}$, Zhuang $P = 0.345$), but no association with hemoglobin concentration (Table 2). To our best knowledge, this is the first identified association between rs7385804 and TS. Effect sizes of these significant associations were consistent in the subsets of population (Table 2).

Common variants in *TMPRSS6* have been reported to be associated with decreased SI, hemoglobin and TS (6,14–16,20). Our evaluation of these iron status parameters confirmed these earlier reports (Table 2). In addition, we found these variants were associated with increased levels of FEP and FEP/hemoglobin ratios in Han^{East} individuals (FEP $P_{rs855791} = 0.007$, FEP/Hb $P_{rs855791} = 3.66 \times 10^{-4}$). Elevated FEP levels and increased FEP/hemoglobin ratios are indicators of erythropoiesis malfunction. In evaluating the correlation between *TMPRSS6* polymorphisms and the risk of anemia, significant associations were found in two subsets of Han individuals (Table 3). The odds ratio (OR) of the rs855791 risk allele was 1.39 (95% CI 1.18–1.64) in Han^{East} ($P = 1.02 \times 10^{-4}$) and 1.47 (95% CI 1.07–2.00) in Han^{North} ($P = 0.016$). The SNP rs4820268 was in linkage disequilibrium with rs855791 ($r^2 = 0.82$ in Han, $r^2 = 0.86$ in Zhuang) and showed similar fashion with rs855791 (Table 3).

Associations of genetic variants with the risk of iron deficiency

There is an overlap in the distribution of hemoglobin concentrations between iron-deficient and iron-sufficient persons (2). And in our study population, a large proportion of women had iron deficiency both in the anemic and control groups (Supplementary Material, Table S2). The *TMPRSS6*-encoding protein Matriptase-2 can modulate the secretion of hepcidin and affect iron absorption in the body (18,19). We identified significant associations between *TMPRSS6* polymorphisms and iron deficiency risk in all three subsets, including Zhuang [OR 1.50 (95% CI 1.32–1.67) in Han, OR 1.69 (95% CI 1.40–1.98) in Zhuang] (Table 4).

Associations between *TMPRSS6* polymorphisms and IDA risk

The results above suggested that *TMPRSS6* polymorphisms might have more direct effects on iron status. We hypothesized that common variants in *TMPRSS6* were primarily associated with anemia caused by iron deficiency. In the following analyses, we tested whether *TMPRSS6* polymorphisms were associated with IDA. The classification of IDA was determined as described in the Materials and Methods. In control groups, individuals with normal hemoglobin levels who exhibited iron deficiency were excluded from further analysis. We found that *TMPRSS6* polymorphisms were strongly associated with the risk of IDA. OR of the rs855791 polymorphism were 1.87 (95% CI 1.65–2.09) in Han and 1.56 (95% CI 1.19–1.92) in Zhuang. Findings with the SNP rs4820268 were similar to that of rs855791 (Table 5). Therefore, rs855791 and rs4820268 in *TMPRSS6* are iron-lowering risk alleles and also genetic risk factors for iron deficiency and IDA.

DISCUSSION

The purpose of this study was to reveal genetic variants associated with the risk of IDA. Specifically, genetic variants in genes involved in iron delivery and hepcidin regulation pathways were selected for analysis. In order to minimize differences caused by gender and age, blood samples from 2139

Table 4. Associations of genetic variants with the risk of iron deficiency

SNP (risk allele)	Gene	Han^{East}	Han^{North}	Zhuang	Han[a]	Han + Zhuang[b]
rs855791 (A)	*TMPRSS6*					
OR		1.48	1.55	1.69	1.50	1.55
95% CI		1.28–1.68	1.19–1.90	1.40–1.98	1.32–1.67	1.40–1.70
P-value		1.16×10^{-4}	0.02	3.60×10^{-4}	2.77×10^{-5}[c]	4.96×10^{-8}[c]
rs4820268 (G)	*TMPRSS6*					
OR		1.49	1.25	1.90	1.43	1.53
95% CI		1.29–1.68	0.89–1.60	1.60–2.19	1.25–1.60	1.38–1.68
P-value		8.89×10^{-5}	0.22	1.92×10^{-5}	2.95×10^{-4}[c]	9.00×10^{-8}[c]
rs3811647 (A)	*TF*					
OR		1.17	0.94	1.10	1.11	1.11
95% CI		0.97–1.38	0.58–1.30	0.85–1.36	0.93–1.29	0.96–1.25
P-value		0.13	0.73	0.44	0.25	0.17
rs7385804 (C)	*TFR2*					
OR		1.15	1.17	0.74	1.15	0.98
95% CI		0.90–1.39	0.739–1.60	0.47–1.02	0.94–1.36	0.81–1.148
P-value		0.27	0.49	0.04	0.20	0.80
rs235756 (G)	*BMP2*					
OR		1.01	0.70	1.31	0.92	1.02
95% CI		0.74–1.28	0.23–1.16	0.95–1.67	0.68–1.15	0.82–1.22
P-value		0.96	0.13	0.15	0.47	0.85

An additive genetic model was assumed and regressions were adjusted for age. Persons with CRP > 10 mg/l were excluded.
[a]Combined results using meta-analysis of Han^{East} and Han^{North}.
[b]Combined results using meta-analysis of Han^{East}, Han^{North} and Zhuang.
[c]*P*-value after Bonferroni correction.

Table 5. Associations of *TMPRSS6* polymorphisms with the risk of IDA

SNP (risk allele)	Han^{East}	Han^{North}	Zhuang	Han[a]	Han + Zhuang[b]
rs855791 (A)					
OR	1.80	2.15	1.56	1.87	1.78
95% CI	1.55–2.05	1.68–2.61	1.19–1.92	1.65–2.09	1.59–1.97
P-value	3.80×10^{-6}	0.001	0.02	1.11×10^{-7}[c]	8.43×10^{-9}[c]
rs4820268 (G)					
OR	1.67	1.89	1.75	1.72	1.72
95% CI	1.42–1.92	1.42–2.37	1.38–2.11	1.50–1.93	1.54–1.91
P-value	4.75×10^{-5}	0.008	0.003	7.05×10^{-6}[c]	6.49×10^{-8}[c]
rs7385804 (C)					
OR	1.00	1.02	0.73	1.00	0.90
95% CI	0.70–1.29	0.49–1.55	0.37–1.09	0.74–1.26	0.69–1.11
P-value	0.98	0.95	0.09	0.99	0.33

An additive genetic model was assumed and regressions were adjusted for age.
Persons with CRP > 10 mg/l were excluded.
Persons with normal hemoglobin levels but iron deficiency were excluded.
[a]Combined results using meta-analysis of Han^{East} and Han^{North}.
[b]Combined results using meta-analysis of Han^{East}, Han^{North} and Zhuang.
[c]*P*-value after Bonferroni correction.

women aged 50–70 years old were collected and further divided into three subsets according to the geographical and ethnic origins.

Of the iron delivery molecules, the *TF* gene was selected for evaluation to determine its association with anemia risk. Several severe mutations in *TF* lead to atransferrinemia, a type of severe microcytic anemia (22). Less severe mutations in *TF* have been shown to be risk factors for IDA (23,24). GWA studies conducted in recent years revealed that SNP rs3811647 in *TF* was significantly associated with increased serum transferrin and TIBC (6,7,14). Therefore, we examined its impact on the risk of anemia. Here, we confirmed previous results showing rs3811647 associations with increased serum transferrin and TIBC in our populations (Table 2). Despite the strong associations of rs3811647 with TIBC and serum transferrin, we did not find associations with the hemoglobin level, iron deficiency or anemia (Tables 3 and 4). We demonstrated a weak association (nominal *P*-values) with SI levels as previously reported (14) (Table 2). Together, these data suggest that rs3811647 generates local effects on the properties of transferrin but limited effects on whole-body iron metabolism status.

Hepcidin, a liver-derived 25-amino acid peptide (25), regulates iron absorption and iron recycling through induced degradation of ferroportin1 in the intestine and in macrophages, respectively (26). Reductions in hepcidin secretion caused by genetic mutations lead to excessive iron absorption and eventually result in hemochromatosis (27). TfR2 is a homologue of the type-1 transferrin receptor (TfR1), but has a lower affinity for transferrin (28). Mutation of *TFR2* leads to hemochromatosis due to decreased hepcidin secretion; therefore, TFR2 is believed to be involved in the hepcidin regulation pathway (27). *TFR2* polymorphisms have been reported to be associated with red blood cell count, mean cell volume and SI levels (12,16,20). We confirmed the association of the rs7385804 *TFR2* polymorphism with decreased SI in the Chinese Han population (Table 2). Lower TS was also found to be associated with rs7385804, but decreased serum transferrin levels and TIBC were not found (Table 2). TS was calculated by dividing the SI content by TIBC, so it is possible that the association of *TRF2* with TS was mainly due to its effect on SI levels. But *TFR2* polymorphism showed a weaker effect on SI than *TMPRSS6* polymorphisms did (Table 2). In addition, no association with iron-deficiency risk was found (Table 4). A previous study reported that the *TFR2* polymorphism had a marginal association with *TFR2* expression levels (12), but the association with hepcidin was unknown. It is possible that the *TFR2* polymorphism was not significantly associated with hemoglobin levels and the risk of anemia because the change in hemoglobin level is not sensitive as SI response to hepcidin regulation.

BMP2 induces hepcidin expression through the BMP co-receptor hemojuvenlin (Hjv) (29). A polymorphism in *BMP2* was reported to be associated with serum ferritin levels in persons with iron overload (11,21). Possibly because of the difference in serum ferritin levels between iron-burdened and anemic persons, we failed to replicate these previous results in our study. Further, we did not find any association between rs235756 in *BMP2* with iron deficiency or anemia risk. Recent studies focused on the hepcidin regulation pathway indicated that BMP6 is a key regulator of hepcidin *in vivo* (30) However, GWA studies did not find any association of iron traits with polymorphism in BMPs either.

TMPRSS6, the gene encoding protein matriptase-2, has an inhibitory effect on the production of hepcidin through cleavage of the BMP co-receptor, hemojuvelin (31). *Tmprss6* mutations in mice lead to increased hepcidin levels and reduced iron absorption (18,19). Human *TMPRSS6* mutations lead to IRIDA (4). Further, these mutations in *TMPRSS6* resulted in loss of function or altered activity of matriptase-2 in IRIDA patients (4,32,33). Polymorphisms in *TMPRSS6* were also found to be associated with a variety of iron traits, including lower SI, hemoglobin, mean cell volume and TS levels (6,13–16,20,34). A recent study indicated that *TMPRSS6* SNP rs855791, a non-synonymous SNP encoding protein matriptase-2^{736A}, generated a stronger inhibitory effect on hepcidin levels than matriptase-2^{736V} (17). Another study also found a suggestive association of *TMPRSS6* polymorphisms with hepcidin mRNA expression levels and urine hepcidin levels (12). We identified significant distribution differences of the matriptase-2^{736V} substitution between normal persons and those with anemia. The distribution of matriptase-2^{736V}, the less inhibitory form, showed higher allelic frequencies and homozygous ratios in persons with anemia (Supplementary Material, Table S1), which suggested elevated hepcidin levels in anemic persons. Further, these distribution differences were significantly associated with an increased risk of anemia in Chinese Han individuals (Table 3). Consistent with elevated hepcidin levels, rs855791 was also associated with lower hemoglobin, SI and TS levels (Table 2). We found it was also associated with elevated FEP and FEP/Hb levels in the Han^{East} population, and that FEP and FEP/Hb levels were higher in people with anemia (Table 1).

In subsequent analyses, we found that polymorphisms of *TMPRSS6* were also significantly associated with iron-deficiency risk in all subsets of our study population (Table 4). This further suggested that altered activity of matriptase-2 influenced iron status. Because various conditions lead to anemia, but upregulation of hepcidin directly leads to decreased iron absorption from the intestine and decreased iron recycling from macrophages, it is not surprising that *TMPRSS6* polymorphisms showed higher OR than the anemia risk (Table 4). Severe iron deficiency will eventually lead to anemia; therefore, we assessed the effects of *TMPRSS6* polymorphisms on the risk of IDA. *TMPRSS6* polymorphisms showed stronger effects on the risk of IDA than on the risk of anemia or iron deficiency alone (Table 5).

In conclusion, our study highlights the importance of matriptase-2 function and the contribution of *TMPRSS6* polymorphisms to the pathological status of persons with iron deficiency and anemia. We identified that *TMPRSS6* polymorphisms are not only associated with lower SI and hemoglobin levels, but are also genetic risk factors for iron deficiency and IDA. Our findings also further supported the role of functional SNP rs855791 in iron metabolism (17). We hypothesized that the altered inhibitory effect of matriptase-2 on hepcidin secretion was possibly the underlying mechanism of the increased risk of iron deficiency and IDA. SNPs in *TF* and *TFR2*, although associated with several important iron traits, did not show significant effects on iron deficiency or anemia risk. Therefore, only *TMPRSS6* polymorphisms, which generate potential effects on hepcidin production, were significantly associated with iron deficiency and IDA in elderly women from Chinese Han and Zhuang populations. Notably, we observed iron deficiency in a large proportion of the elderly Chinese women within our study population. This trend is also apparent in the control individuals, reaching levels of 35% (Supplementary Material, Table S2). Currently, the precise underlying mechanisms are undefined. Considering the complexity of the regulatory network required to maintain iron homeostasis, collaborative efforts will be required to further dissect the risk factors for initiation and progression of disease. Our study, for the first time, sheds light on the link between genetic variants, including *TMPRSS6,* and the high risk for IDA in a large cohort of the Chinese population. These findings may pave the way for further understanding of the functional roles of *TMPRSS6* in other diseases related to iron deficiency.

MATERIALS AND METHODS

Study populations and sample collection

A total of 2139 women (50–70 years old) from three separate regions in China were involved in this study, which was originally for the purpose of studying nutrition and health in China. All anemic subjects and the control subjects were selected from this population.

Each participant was interviewed and a basic health examination was done. Those with serious diagnosed diseases were excluded from our study. After an overnight fast, venous blood samples were collected and questionnaires on diet, life style, medical history and physical activity were completed. Samples from Jiangsu and Shanxi Province are Han people, and samples from the Guangxi Zhuang Autonomous Region are Zhuang people. We grouped samples into three subsets according to the geographic location and ethnic origin (Supplementary Material, Fig. S1). Therefore, in the following statistical analyses, samples from the Jiangsu Province are called Han^{East}. Samples from Shanxi Province are called Han^{North}. Samples from Guangxi Zhuang Autonomous Region are called Zhuang. Detailed numbers of case and control samples are listed in Table 1. Written informed consent was obtained from all participants and study protocols were approved by the Institute Review Board of Institute of Nutrition and Food Safety and Institute for Nutritional Sciences.

Hematological parameters

The following hematological parameters were measured: hemoglobin, SI, serum ferritin, serum transferrin, FEP and TIBC. Blood hemoglobin concentration was measured by finger-prick. The Ferrozine method was used to measure SI (Randox Life Sciences). We used the immunoturbidimetry method to detect serum ferritin, serum transferrin and C-reactive protein (CRP) levels (Randox Life Sciences). FEP was measured by hematofluorometer (Hitachi F-4000). TIBC was measured using the colorimeter-based method (Randox Life Sciences). TS was calculated from SI and TIBC (TS = SI/TIBC × 100). Genomic DNA was extracted from peripheral blood leukocytes using commercial blood DNA extraction kits (TIANamp Blood Genomic DNA Purification Kit) according to the manufacturer's protocol.

Classification of iron status

Anemia was defined as hemoglobin concentration <120 g/l according to the criteria defined by WHO. Control samples were selected from women volunteers with hemoglobin (Hb) levels of >130 g/l. Case samples were women with Hb levels <120 g/l. A total of 1023 women were anemic and 1116 were normal. According to standards from a previous report (35), persons with one of the following were defined as iron deficient: TS $<$ 16%, serum ferritin $<$ 15 ng/ml and FEP/hemoglobin ratio >3.0 μg/g. People with both iron deficiency and anemia were identified as having IDA. Because chronic inflammation and infectious diseases affect body iron absorption and lead to disturbed iron status (36), people with CRP $>$ 10 mg/l were excluded from the iron deficiency and IDA classification.

Genotyping

The SNPs rs3811647, rs7385804, rs235756, rs855791 and rs4820268 were genotyped using 40× Taqman SNP Genotyping Assay (Applied Biosystems). Polymerase chain reaction amplification was performed using 40× Taqman SNP Genotyping Assay, 2× Taqman Genotyping Master Mix (Applied Biosystems) and ddH_2O up to 5 μl of final volume per well. After amplification, fluorescent detection was completed on ABI PRISM 7900HT Sequence Detection System, using SDS 2.3 software for allele discrimination. Samples of 5% were replicated and the concordance rate was above 99%.

Statistical analysis

The linear regression model was used to analyze the association between genetic variants and hematological parameters. The additive genetic model was used to measure the association with hematological parameters and all associations were adjusted for age. Coefficients (beta) and the standard errors (SE) of the linear regressions are listed in Table 2. Serum ferritin levels were $\log_{10}$-transformed. To determine the association of variants with anemia, iron deficiency and IDA, logistic regression was used. Association with anemia, iron deficiency and IDA was adjusted for age. The additive genetic model was assumed. The OR was calculated from the exponentiated beta coefficient of logistic regression. For the Hardy–Weinberg equilibrium test, we used a method published by Wigginton *et al.* (37). Statistical analyses of linear regression and logistic regression were performed using R (http://www.r-project.org/). Linkage disequilibrium was analyzed using the genetics package of R (http://cran.r-project.org/web/packages/genetics/). Meta-analysis was performed using METAL (http://www.sph.umich.edu/csg/abecasis/Metal/).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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