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Genetic polymorphisms in bovine *transferrin receptor 2* (*TFR2*) and *solute carrier family 40* (*iron-regulated transporter*), *member 1* (*SLC40A1*) genes and their association with beef iron content

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Summary

Beef is considered to be an excellent source of dietary iron. However, little is known about the genetic control of beef iron content. We hypothesized that genetic polymorphisms in *transferrin receptor 2* (*TFR2*) and *solute carrier family 40* (*iron-regulated transporter*), *member 1* (*SLC40A1*) could influence skeletal muscle iron content. The objective of this study was to use Angus cattle to identify single-nucleotide polymorphisms (SNPs) in the exons and flanking regions of the bovine *TFR2* and *SLC40A1* genes and to evaluate the extent to which genetic variation in them was associated with bovine longissimus dorsi muscle iron content. Ten novel SNPs were identified in *TFR2*, of which one SNP tended to be associated ($P < 0.013$) with skeletal muscle iron content. Nine novel SNPs in *SLC40A1*, NC007300: rs133108154, rs137140497, rs135205621, rs136600836, rs134388440, rs136347850, rs134186279, rs134621419 and rs137555693, were identified, of which SNPs rs134388440, rs136347850 and rs137555693 were significantly associated ($P < 0.007$) with skeletal muscle iron content. High linkage disequilibrium was observed among *SLC40A1* SNPs rs134388440, rs136347850 and rs137555693 ($R^2 > 0.99$), from which two haplotypes, TGC and CAT, were defined. Beef from individuals that were homozygous for the TGC haplotype had significantly ($P < 0.001$) higher iron content than did beef from CAT homozygous or heterozygous individuals. The estimated size of effect of the identified haplotypes was 0.3% of the phenotypic variance. In conclusion, our study provides evidence for genetic control of beef iron concentration. Moreover, SNPs identified in *SLC40A1*, rs134388440, rs136347850 and rs137555693 might be useful markers for the selection of Angus cattle for altered iron content.

Keywords bovine, ferroportin 1, iron, longissimus dorsi, single-nucleotide polymorphism, SNP, *SLC40A1*, *solute carrier family 40* (*iron-regulated transporter*), member 1, *TFR2*, *transferrin receptor 2*.

Introduction

Iron is required as an ionic cofactor for a variety of proteins and functions as an essential biological electron donor and acceptor (Edison *et al.* 2008). However, iron deficiency anaemia is still a major public health problem worldwide (Andrews 2008). Red meat is a good source of dietary iron in regard to both amount and bioavailability, particularly beef, which contains the highest amount of iron compared with other meat sources consumed in the United States (Charpenter & Clark 1995). However, on average a 112 g serving size of beef would only supply ~11% of the average daily requirement of a woman (Food and Nutrition Board 2001).

As a redox-active transition metal, iron, although important physiologically, is toxic at high concentrations, where it generates reactive oxygen species (Galaris *et al.* 2008). Iron homoeostasis thus must be maintained systemically by the

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rate of iron absorption, iron release and the rate of iron utilization. Transferrin receptor 2 (encoded by *Transferrin receptor 2*, *TFR2*) and ferroportin 1 (encoded by *solute carrier family 40* (*iron-regulated transporter*), *member 1*, *SLC40A1*) are two important proteins in the regulation of body iron homoeostasis. As there is no paracellular iron transport under normal circumstances, iron requires the assistance of transporter proteins to cross the cell membrane. TFR2 mediates cellular uptake of transferrin-bound iron (Gao *et al.* 2009), and SLC40A1 is the only known iron exporter that facilitates iron transport out of a cell (Abboud & Haile 2000; Donovan *et al.* 2000; McKie *et al.* 2000). Moreover, it is proposed that TFR2 may sense the concentration of diferric transferrin in blood and then positively regulate hepcidin expression. Hepcidin is a peptide hormone that acts as the central regulator of serum iron concentration, and its release leads to decreased serum iron levels (Gao *et al.* 2009). In addition, SLC40A is critical for maintaining body iron homoeostasis by being a receptor of hepcidin. The binding of hepcidin to SLC40A1 leads to SLC40A1 internalization and degradation and consequently decreases iron efflux into the plasma (De Domenico *et al.* 2007).

Iron content in beef exhibits a large amount of natural variation (Gerber *et al.* 2009). It is known to vary with physiological and environmental factors, such as animal age, muscle type (Doornenbal & Murray 1981), diet (Shenk *et al.* 1934) and interaction between minerals (Anke *et al.* 1970). Variation in serum and hepatic iron concentration among inbred strains of mice has been reported, which indicates that differences in iron content may be heritable (Leboeuf *et al.* 1995; Dupic *et al.* 2002; Bayele & Srai 2009). Strain-to-strain differences have also been observed in duodenal mRNA expression of *SLC40A1* (Dupic *et al.* 2002). Influence from genetic factors is also indicated by inherited disorders such as hereditary hemochromatosis, which is characterized by systemic iron overload (Le Gac & Ferec 2005). Several variants in genes that encode for iron-related proteins, for example, *HFE*, *hemochromatosis type 2* (*juvenile*), *TFR2* and *SLC40A1*, have been reported to cause hereditary hemochromatosis. Mutations in human *TFR2* and *SLC40A1* cause two types of iron overload disorders, which are referred to as type III (Camaschella *et al.* 2000) and type IV hereditary hemochromatosis (Pietrangelo 2004), respectively. To our knowledge, no single nucleotide polymorphisms (SNPs) have yet been reported for *TFR2* or *SLC40A1* in cattle. Moreover, skeletal muscle, which contains a large proportion of body iron, is of great interest regarding iron homoeostasis (Robach *et al.* 2007). However, little is currently known about the genetic factors that may influence iron storage in cattle, especially in skeletal muscle.

Growing evidence shows that polymorphisms in genes that encode a component of the iron regulation network can potentially influence whole-body iron content. TFR2 and ferroportin 1 play an important role in the maintenance of iron homoeostasis. Therefore, we hypothesized that the *TFR2* and *SLC40A1* genes would be candidates for conferring heritable differences in iron content of skeletal muscle on individuals, and that these SNPs might be useful as markers for the variation in iron content in beef. In this study, we identified ten novel SNPs in *TFR2* and nine in *SLC40A1* and evaluated their relationship with iron content of LD muscle in Angus cattle.

Materials and methods

Animals and sample collection

American Angus sired cattle (n = 1086) were used in this study. The cattle used were 391 young bulls, 181 steers and 154 heifers from the Iowa State University Angus breeding project, raised in Iowa, plus 360 steers from a collaborating herd in California. Animals were harvested at commercial facilities at an average age of 457 ± 46 days. Longissimus dorsi muscle samples were collected, trimmed of external connective tissue and adipose tissue, freeze ground, packed, and stored at −20 °C until iron concentration analysis.

Total, heme, and non-heme iron concentration analysis

Beef samples were dried, and moisture was determined according to the AOAC official method 934.01 (2005). Dry samples were subjected to closed-vessel microwave digestion according to AOAC official methods 999.10 (Jorhem & Engman 2000). Total iron concentration in the beef was determined using inductively coupled plasma-optical emission spectroscopy (ICP-OES, SPECTRO Analytical Instruments). Non-heme iron content in beef was measured according to Rebouche *et al.* (2004). Heme iron concentration was calculated by subtracting non-heme concentration from total iron concentration.

DNA polymorphism identification and genotyping

Genomic DNA was purified by standard phenol chloroform methods. Six DNA samples, half from cattle with high muscle iron and half from cattle with low muscle iron, were selected for SNP identification. Each of eight pairs of PCR primers was designed to amplify the exons and their adjacent intronic regions in *TFR2* and *SLC40A1* (Tables S1 and S2). PCR was carried out in a DNA engine thermal cycler (Bio-Rad) with the following protocol: 94 °C for 5 min; followed by 39 cycles of 94 °C for 30 s; 58 °C for 35 s, and 72 °C for 35 s; with a final extension step at 72 °C for 5 min. PCR products were purified using ExoSAP-IT® (USB). The DNA sequences of PCR amplicons were determined with an ABI 3730 DNA Analyzer (Applied Biosystems Inc.) at the Iowa State University DNA Facility. The genotypes of the identified SNPs in the 1086 Angus cattle

were determined by Sequenom® at the Genomic Technologies Core Facility at Iowa State University.

Statistical analysis

Data were analysed using the following mixed linear model (PROC MIXED; SAS Inst., Inc.):

$$Y_{ijklm} = \mu + S_i + C_j(S_i) + A_k + G_1 + G_1 \times S_i + \text{Sire}_m + e_{ijklm},$$

Where: Y_{ijklm}, dependent variable (beef iron concentration; μg per gram of wet weight of LD muscle); μ, overall mean; S_i, fixed effect of the ith level of source (i = 1, 2); C_j (S_i), fixed effect of the jth level of contemporary group (contemporary group was defined as: harvest date and sex within harvest date) nested with in ith level of source (j = 1–19); A_k, age, in days, of the kth observation as a covariate (k = 1–1086); G_l, fixed effect of the lth level of genotype in the genotype as class effect model (l = 1, 2, 3), or G_l, genotype (homozygote AA = −1, heterozygote AB = 0, and homozygote BB = +1) as a covariate of the lth observation in an allele substitution model; $G_l \times S_i$, interaction term of the lth genotype with the ith source; Sire_m, random effect of sire m; $\text{Sire}_m \sim N(0, \sigma_s^2)$; e_{ijklm}, random error term; $e_{ijklm} \sim N(0, \sigma_e^2)$.

Significance threshold correction for multiple comparisons was determined based on the correlation and dependence among SNPs to estimate the number of independent tests within a gene (Cheverud, 2001). The number of estimated independent tests used for correction was 7.5377 for *TFR2* and 7.1083 for *SLC40A1*. Least square means (±SE) were determined using the genotype as a class effect as defined earlier. These values were compared using pairwise t-tests. The additive and dominant genetic effect of each locus was estimated according to Khatib *et al.* (2007). The haplotype and linkage disequilibrium were analysed using Haploview (Barrett *et al.* 2005).

Results

DNA polymorphism identification and selected nucleotide sequence alignment

The bovine *TFR2* gene is 11243 bp in length and is located on chromosome 25. We amplified and sequenced the exons of *TFR2* and their flanking regions in six Angus cattle, and ten nucleotide polymorphisms were identified. These were NC_007326.4: rs133534046, rs137342277, rs110312059, rs135204935, rs133610571, rs137409353, rs110341742, rs132876454, rs134140507 and rs134923607. Three SNPs were found within *TFR2* exons, none of which changed the amino acid sequence.

The bovine *SLC40A1* gene is located on chromosome 2. The gene is 23,683 bp in length, with nine exons. In this study, nine nucleotide substitutions were identified. They were NC_007300.3: rs133108154, rs137140497, rs135205621, rs136600836, rs134388440, rs136347850, rs134186279, rs134621419 and rs137555693. Two SNPs, rs134186279 and rs137555693, were located in the exons. SNP rs134186279 in exon 7 was predicted to result in an amino acid replacement from methionine (ATG) to valine (GTG), and the other sequence variant, rs137555693 in exon 8, was synonymous. The rest of the SNPs were in intronic regions, four of which, rs135205621, rs134388440, rs136347850 and rs134621419, were located close to exons. Multiple sequence alignments were carried out using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html) for rs135205621, rs134388440, rs136347850 and rs137555693 among cattle, human, chimpanzee, monkey, house mouse and rat (Fig. 1). These four polymorphisms were found to be conserved among these species.

Genotype frequencies of the identified SNPs

Angus sired cattle (n = 1086 head) were genotyped for the identified SNPs. Four of the SNPs in *TFR2*, rs110312059, rs110341742, rs134140507 and rs134923607, were in Hardy–Weinberg equilibrium ($P > 0.05$, Falconer & Mackay 1996), and the rest of the ten SNPs were not ($P < 0.05$, Table 1). High linkage disequilibrium was observed among SNPs rs133610571, rs137409353, rs132876454, rs134140507 and rs134923607 ($R^2 > 0.50$).

Most of the SNPs identified in *SLC40A1* were in Hardy–Weinberg Equilibrium ($P > 0.05$, Falconer & Mackay 1996), except for rs135205621 and rs136347850. SNPs rs133108154, rs137140497, rs135205621 and rs136600836 showed a low minor allele frequency (Table 2). Near complete linkage disequilibrium was observed among SNPs rs134388440, rs136347850 and rs137555693 ($R^2 > 0.99$). These defined two haplotypes, TGC and CAT, with frequency 61.1% and 38.6%, respectively (data not shown).

Associations between the identified SNPs and iron content of LD muscle

We tested for the association between the identified SNPs and skeletal muscle iron content. As shown in Table 1, only one of the ten SNPs in *TFR2*, rs133610571, had a nominal *P*-value less than 0.05 for the genotypic class effect model. After multiple comparison adjustment, the significance threshold is set at 0.007, while *P*-values between 0.007 and 0.013 were considered to have a statistical tendency. Therefore, rs133610571 was the only SNP that tended to be associated with beef iron content ($P < 0.013$) among the SNPs identified in *TFR2*.

Five SNPs from *SLC40A1* were found to have nominal *P*-values less than 0.05 (Table 2) for both the genotypic class effect and additive effect model. After adjustment for multiple comparisons, *P*-values less than 0.007 were considered to be statistically significant. Three SNPs,

rs135205621
GTCAGCACCTTTTCTCCTGTCCTG**T**TTCCAGGGGGACCGGATGTGGCACT 7194 Cattle
ATCAAAACATTTTCT-CT-TTTCA**T**TTA-AGGGAGATCGGATGTGGCACT 10510 Human
ATCAAAACATTTTCT-CT-TTTCA**T**TTA-AGGGAGATCGGATGTGGCACT 8479 Chimpanzee
ATCAAAACATTTTCT-CT-TTTCA**T**TTA-AGGGAGATCGGATGTGGCACT 9663 Monkey
AACCAAACATCTTTC----TTTTG**T**TTA-AGGGGGATCGGATGTGGCACT 4298 House mouse
AGCCAAACTTCTTTC----TCTTG**T**TTA-AGGGGGATCGGATGTGGCACT 4636 Rat

rs134388440
------GTTAATCAATACACTTGG**T**TTA-TGAGTTGCGTTATGTACGTGG 19232 Cattle
------TCTAATCTATACTCTTGG**T**TTACAGCTTTGTATTGTGTAAATGG 20155 Human
AAAATGTCTAATCTATACTCTTGG**T**TTACAGCTTTGTATTGTATAAATGG 18618 Chimpanzee
------GCTAATCTGTACTCTTGG**T**TTACAGGTTTGTATTGTGTAAATGG 18761 Monkey
------GTTAGTCTGTACTGTTGG**T**TT--AGATTTATAGCACATAAATGA 13068 House mouse
------GATGGTCTGCACTGTTGG**T**TT--AGACTTATAGCACGTAAACAT 13391 Rat

rs136347850
TCTCTTTGATAGATTTGCACACTT**G**CCTGCCTTTTTCACCTATCTCTGTA 19288 Cattle
TCTCTTTGATGGGTTTGCACACTT**A**CCTGCCTCTTTCACCTGCCTCTCTA 20211 Human
TCTCTTTGATGGGTTTGCACACTT**A**CCTGCCTCTTTCACCTGCCTCTCTA 18674 Chimpanzee
----TTTGATGGGTTTGTACACTT**A**CCTGCCTCTTTCACCTGCTTCTCTA 18813 Monkey
TGTAACTCT----------CACTT**A**CCTGCCTCTTGCACCTACCTGTGTA 13112 House mouse
TTTCTTTCACAGTTTGGAACGCTC**A**CTCTCCTCTTTCACCTACTTC--TA 13435 Rat

rs137555693
TGAGCCCTTCCGCACTTTCCGAGA**C**GGATGGGTCTCCTATTACAACCAGT 20505 Cattle
TGAGCCCTTCCGTACCTTCCGAGA**T**GGATGGGTCTCCTACTACAACCAGC 21742 Human
TGAGCCCTTCCGTACCTTCCGAGA**T**GGATGGGTCTCCTACTACAACCAGC 20200 Chimpanzee
TGAGCCCTTCCGTACCTTCCGAGA**T**GGATGGGTCTCCTACTACAACCAGC 20353 Monkey
AGAGCCCTTCCGCACTTTCCGAGA**T**GGATGGGTCTCCTACTATAACCAGC 14220 House mouse
AGAACCCTTCCGCACTTTTCGAGA**T**GGATGGGTCTCCTACTATAACCAGC 14757 Rat

Figure 1 Nucleotide sequence alignments for *SLC40A1* SNPs rs135205621, rs134388440, rs136347850 and rs134621419 among several mammalian species. The multiple sequence alignments were carried out using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The nucleotide polymorphism loci are shown in bold.

rs134388440, rs136347850 and rs137555693, were strongly ($P < 0.007$) associated with iron content, either analysed with genotype as a class effect or as covariate in the allele substitution model. Polymorphism rs136600836 tended ($P < 0.014$) to be associated with iron content when genotype was coded as a class effect (Table 2). Similar association results were obtained for cattle from both Iowa and California. As a result, we present only the combined analysis here.

As total iron in muscle exists in heme and non-heme forms, association analyses were also conducted between identified SNPs and the concentration of the two subtypes of iron (data not shown). Only SNP rs134388440 in gene *SLC40A1* was found to be significantly associated with heme iron concentration after multiple comparison adjustment ($P < 0.007$). SNP rs136347850 in *SLC40A1* tended to be associated with heme iron concentration ($P < 0.014$). No association was found between the identified SNPs and the non-heme iron content ($P > 0.10$).

Moreover, as aforementioned, *SLC40A1* SNPs rs134388440, rs136347850 and rs137555693 were in almost complete linkage disequilibrium ($R^2 > 0.99$) and defined two haplotypes, TGC and CAT (Table 3). Homozygotes with TGC or CAT haplotype had frequency of 39.87% or 16.40%, respectively, and the frequency for the heterozygote was 43.73%. We did an association analysis for haplotype and iron content. The results showed that beef from individuals that were TGC homozygous had significantly ($P < 0.001$) higher iron contents than did beef from CAT homozygous or heterozygous individuals. The estimated size of effect of the identified haplotypes was 0.3% of the phenotypic variance. Additive and dominance effects were tested for this haplotype (Table 3). A significant ($P < 0.005$) additive effect was observed.

Discussion

TFR2 (encoded by *TFR2*) and solute carrier family 40 (iron-regulated transporter), member 1 (encoded by *SLC40A1*) play an important role in regulating body iron homoeostasis. Currently, hundreds of SNPs have been reported in the coding and non-coding regions of human and mouse *TFR2* and *SLC40A1*, but none have been reported for cattle. In this study, ten novel SNPs, NC_007326.4: rs133534046, rs137342277, rs110312059, rs135204935, rs133610571, rs137409353, rs110341742, rs132876454, rs134140507 and rs134923607, were identified in bovine *TFR2*, and nine novel SNPs, NC_007300.3: rs133108154, rs137140497, rs135205621, rs136600836, rs134388440, rs136347850, rs134186279, rs134621419 and rs137555693, were identified in the exons and their flanking regions in bovine *SLC40A1*.

Besides the weak association between the beef iron content and the genetic polymorphisms in *TFR2*, strong associations ($P < 0.007$) were found for beef iron content and SNPs identified in *SLC40A1*. With the growing understanding of the molecular basis of iron regulation, several mutations that are linked with iron overload disorder have been reported in iron-related genes (Cazzola 2003; Pietrangelo 2004). Moreover, polymorphisms in iron-related genes may contribute to body iron variation. Human serum iron status was found to be significantly associated with

Table 1 Associations between *TFR2* SNP genotypes and beef iron content.

SNP	Genotype	*N*	Frequency	Mean[1]	±SE	*P*-value[2]	
						Genotypic class effect model	Additive effect model
rs133534046	GG	498	0.93	13.02	0.20	0.12	0.33
	GT	31	0.06	13.88	0.53		
	TT	3	0.01	N.A.	N.A.		
rs137342277	AA	34	0.09	N.A.	N.A.	0.48	0.78
	GA	314	0.80	12.87	0.21		
	GG	42	0.11	13.08	0.60		
rs110312059	CC	25	0.02	12.30	0.50	0.12	0.14
	CT	275	0.28	13.14	0.19		
	TT	692	0.70	13.29	0.15		
rs135204935	GG	214	0.61	13.39	0.26	0.56	0.56
	GA	135	0.39	13.20	0.25		
	AA	0	0	N.A.	N.A.		
rs133610571*	CC	269	0.39	13.01	0.20	0.012	0.27
	CT	364	0.53	13.24	0.19		
	TT	54	0.08	12.03	0.39		
rs137409353	AA	38	0.05	12.22	0.43	0.05	0.20
	CA	195	0.27	13.30	0.21		
	CC	485	0.68	13.22	0.17		
rs110341742	GG	598	0.97	13.09	0.17	0.81	0.81
	AG	16	0.03	13.25	0.72		
	AA	0	0	NA	NA		
rs132876454	GG	444	0.54	13.21	0.18	0.08	0.27
	GT	342	0.42	13.24	0.19		
	TT	30	0.04	12.11	0.50		
rs134140507	AA	747	0.70	13.19	0.15	0.09	0.15
	GA	296	0.27	12.16	0.47		
	GG	30	0.03	13.13	0.19		
rs134923607	AA	27	0.03	11.99	0.49	0.07	0.25
	CA	281	0.30	13.09	0.16		
	CC	614	0.67	13.09	0.19		

[1]Values are expressed as μg per g of wet weight of LD muscle.

[2]*P*-values were not corrected for multiple comparisons.

*P-value <0.013 (adjusted significance threshold, $P = 0.007$; statistical tendency, $0.007 < P < 0.013$).

polymorphisms in genes that encode bone morphogenetic protein 2 (Milet *et al.* 2007), duodenal cytochrome b (Constantine *et al.* 2009), transferrin (Benyamin *et al.* 2009b), transmembrane protease, and serine 6 (Benyamin *et al.* 2009a). No association between iron variation in muscle and *TFR2* or *SLC40A1* gene has been reported. To our knowledge, this is the first study addressing the association between genetic polymorphisms in *TFR2* and *SLC40A1* and variation in muscle iron content.

The three SNPs in gene *SLC40A1* that were found to be significantly associated ($P < 0.007$) with beef iron content, rs134388440, rs136347850 and rs137555693, were further investigated. SNPs rs134388440 and rs136347850 were located −81 and −26 nt upstream of exon 7, respectively. SNP rs137555693, located in exon 8, was not predicted to change the protein sequence. Its location, however, was notable because exon 8 encodes an important domain of *SLC40A1*. In humans, this domain bears the hepcidin binding sites (residue 324–343), phosphorylation sites (p.Y301 and p.Y302) and ubiquitination site (p.K253). Sequence alignment of this domain showed high similarity between humans and cattle (data not shown). As shown in Fig. 1, rs134388440, rs136347850 and rs137555693 were in conserved sequences. It has been suggested that similarities in sequences between divergent organisms imply functional constraint. Therefore, the result from the nucleotide sequence alignment may indicate a functional role for rs134388440, rs136347850 and rs137555693.

The efficiency of mRNA splice site recognition is under the combinatorial control of several parameters, such as splice site strength, the presence or absence of splicing enhancers and silencers, and RNA secondary structures. Sequence variants that affect these parameters may potentially influence pre-mRNA processing and consequently

Table 2 Associations between *SLC40A1* SNP genotypes and beef iron content.

SNP	Genotype	*N*	Frequency	Mean[1]	±SE	*P*-value[2]	
						Genotypic class effect model	Additive effect model
rs133108154	AA	479	0.94	13.42	0.21	0.48	0.79
	AG	31	0.06	13.54	0.52		
	GG	1	0.00	N.A.	N.A.		
rs137140497	AA	802	0.76	13.25	0.15	0.10	0.09
	GA	237	0.23	12.81	0.21		
	GG	15	0.01	13.39	0.75		
rs135205621	CC	25	0.03	13.55	0.64	0.02	0.011
	CT	216	0.21	13.59	0.22		
	TT	779	0.76	13.03	0.15		
rs136600836*	CC	16	0.01	11.15	0.74	0.013	0.04
	CG	272	0.25	13.04	0.20		
	GG	824	0.74	13.26	0.14		
rs134388440**	CC	157	0.15	12.69[a]	0.25	<0.001	<0.001
	TC	486	0.46	12.92[a]	0.16		
	TT	412	0.39	13.53[b]	0.17		
rs136347850**	AA	161	0.15	12.79[a]	0.25	0.002	<0.001
	GA	457	0.43	13.00[a]	0.17		
	GG	437	0.42	13.54[b]	0.17		
rs134186279	AA	139	0.19	13.20	0.27	0.93	0.75
	AG	362	0.49	13.18	0.21		
	GG	234	0.31	13.10	0.24		
rs134621419	CC	117	0.22	13.26	0.29	0.56	0.69
	CT	266	0.49	13.58	0.24		
	TT	157	0.29	13.42	0.29		
rs137555693**	CC	377	0.36	13.54[a]	0.17	0.002	<0.001
	CT	503	0.48	12.95[b]	0.16		
	TT	167	0.16	12.77[b]	0.25		

[1]Values are expressed as μg per g of wet weight of LD muscle.
[2]*P*-values were not corrected for multiple comparisons.
[a,b]Values in each single nucleotide polymorphism (SNP) with different superscripts differ at $P < 0.005$.
**P*-value <0.014 (adjusted statistical tendency, $0.007 < P < 0.014$).
***P*-value <0.007 (adjusted significance threshold, $P = 0.007$).

Table 3 Effect of haplotypes of genotype *SLC40A1* rs134388440, rs136347850 and rs137555693 on LD muscle iron content, and estimates (±SE) of additive and dominance effects associated with the haplotypes.

Genotype			Number of animals	Genotype frequency (%)	Iron content[1]
rs134388440	rs136347850	rs137555693			
TT	GG	CC	372	39.87	13.51[A] ± 0.16
CT	AG	CT	408	43.73	13.00[B] ± 0.15
CC	AA	TT	153	16.40	12.80[B] ± 0.23
R^2			0.003		
Haplotype additive effect			0.38 ± 0.12***		
Haplotype dominance effect			−0.18 ± 0.17		

[1]Values are expressed as LSmeans ± SE. Iron content is expressed as μg per gram of wet weight of LD muscle.
[2]Proportion of phenotypic variance explained by the haplotype.
[A,B]Values with different superscripts differ at $P < 0.001$.
***$P < 0.005$.

protein expression (Hertel 2008). Exonic splicing enhancers (ESEs) are *cis*-acting RNA sequence elements located within exons that can increase exon inclusion by serving as binding sites for the assembly of multicomponent splicing enhancer complexes (Black 2003). With online bioinformatics tool ESEfinder (Cartegni *et al.* 2003; Smith *et al.* 2006), SNP rs137555693 was predicted to be in a putative alternative splicing factor/splicing factor 2 (ASF/SF2) binding site (GAGACGG). Thus, it is possible that SNP rs137555693, even though it did not cause amino acid change, would influence the function of *SLC40A1* via interference with splicing efficiency of exon 8 as a part of ESE.

SNPs rs134388440 and rs136347850 were located at the 5′ end of exon 7. With an online alternative splice site predictor (Wang & Marin 2006), SNP rs136347850 was found to be a few nucleotides away from a putative RNA splicing site. In addition, this polymorphism was not in Hardy–Weinberg equilibrium, which potentially indicates selection at this site. Mammalian RNA splicing is also known to be regulated by cis-acting elements, either as enhancer or silencer. In the human *amyloid beta* (*A4*) *precursor protein* (*APP*) gene, Shibata *et al.* (1996) identified two cis-acting elements, ATGTTT and TTT, involved in the modulation of RNA splicing of this gene. The two cis-elements found were at the 5′ end of an exon, one as enhancer and the other one as silencer. In the current study, the rs134388440 locus was in a TTT sequence that might be involved in a cis-acting element, hence potentially regulating the efficiency of intron excision. It is also reported that secondary structure, loop or stem motifs may be part of the 'mRNA splicing code' that determines exon recognition (Hiller *et al.* 2007). As TTT is a stem-forming sequence, nucleotide replacement within this sequence would disrupt the pre-mRNA secondary structure, which might consequently affect the mRNA splicing.

Thus, these polymorphisms in *SLC40A1* might influence the iron content of muscle by affecting the production of biologically active protein as a result of interference with the efficiency of mRNA splicing recognition. Although there is currently no evidence supporting the role for the three SNPs in *SLC40A1* expression, their possible involvement cannot be ruled out. High linkage disequilibrium observed for these three SNPs may be another explanation for the strong associations. It is also possible that they are in linkage disequilibrium with a causative alteration that has not yet been identified.

In conclusion, ten novel SNPs were identified in the *TFR2* gene and nine in the *SLC40A1* gene. Three of the SNPs in *SLC40A1*, rs134388440, rs136347850 and rs137555693, were strongly associated with beef iron content, which provides evidence for genetic control of beef iron concentration. Moreover, these SNPs might be useful markers for selecting for altered muscle iron content. Further investigations are needed to verify the observed effect in other independent cattle populations and to elucidate the biological mechanisms of the SNP effect. Furthermore, these results indicate that iron content of beef may be a good model for studying the genetic control of iron content, and this may help to elucidate the genetic mechanisms underlying diseases such as hemochromatosis.

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Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Primer sequences for *TFR2*.

Table S2 Primer sequences for *SLC40A1*.

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