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Short Communication

Association of SNPs in transferrin and transferrin receptor genes with blood iron levels in human

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ABSTRACT

Iron is bound to mobile transferrin (TF) and ferritin in blood. TF receptors (TFRC and TFR2) regulate intracellular iron by delivering iron from TF into the cytoplasm. In this study, we examined the effects of 10 single nucleotide polymorphisms (SNPs) in each of the genes for TF and TF receptors on blood iron concentrations in Japanese subjects. Blood iron levels were determined by microwave plasma-atomic emission spectrometry and the SNPs were analyzed by polymerase chain reaction followed by restriction fragment length polymorphism analysis. Blood iron levels in males were significantly higher than those in females. Therefore, the analysis was performed only in males. Blood iron concentrations did not correlate with age and postmortem intervals in males. Among the 10 SNPs in *TF*, *TFRC*, and *TFR2* genes, significant associations were observed between TF genotypes (rs12769) and male iron concentrations. Individuals with genotype GG in rs12769 had significantly higher blood iron concentrations than those with GA. Previous studies have shown the association between high tissue iron concentrations and disease, liver iron levels are higher in infants dying from sudden infant death syndrome and decreased blood iron concentrations were observed in critically ill children. Therefore, rs12769 in *TF* might be related to diseases and mortality risk.

1. Introduction

Iron is the most abundant metal in biological tissues. It is crucial for oxygen transport and storage, electron transport and energy metabolism, antioxidant and beneficial pro-oxidant functions, and oxygen sensing. However, excess iron is toxic because of the generation of free radicals [1], and cause dysfunction, fibrogenesis and carcinogenesis in organs [2]. Therefore the processes regulating iron homeostasis and iron absorption are critical. Moreover, an association between high tissue iron concentrations and disease (infection, neoplasia and ischaemic heart disease) has been reported [3] and elevated serum iron associates with liver diseases [4].

In blood, iron is bound to mobile transferrin (TF) and ferritin. TF receptor (TFRC, also known as TFR1) and transferrin receptor 2 (TFR2) are highly homologous type II transmembrane proteins that regulate intracellular iron by delivering iron from TF into the cytoplasm [5]. TFRC is ubiquitously expressed, whereas TFR2 is highly expressed in hepatocytes and to a lesser extent in erythrocytes and the duodenum [6]. Recent studies have shown that single nucleotide polymorphisms (SNPs) in iron homeostasis genes have a functional impact on both ferritin and TF levels [7].

The genetic background of iron retention is currently not fully understood. Therefore, this study sought to elucidate the genetic background of iron retention through a comprehensive investigation into the association of SNPs in iron homeostasis genes and blood iron levels in Japanese subjects.

2. Materials and methods

2.1. Study population

Postmortem blood samples (n = 102: 66 males and 36 females) from Japanese subjects at Shimane University were collected within 48 h since deaths. Samples were selected from subjects with external cause deaths, those with certain diseases (infection, neoplasia and ischaemic heart disease; these are shown to be related to anemia) were not included. Genomic DNA was extracted from blood samples using a QIAamp DNA Blood Mini Kit (Qiagen N.V., Venlo, Netherlands). The study protocol, including the use of biological samples derived from autopsy cases, was reviewed and approved by the Human Ethics Committee of Shimane University School of Medicine.

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Table 1
Primer sequences, annealing temperatures, and restriction enzymes for PCR-based genotyping of TF, TFRC, and TFR2.

Gene		Primer	Sense/ antisense	Sequence	Annealing temp (°C)	Product size (bp)	Restriction enzyme
TF							
rs12595 (A to G)	Intron variant	rs12595 F rs12595 R	Sense Antisense	3′-GAGCAGCACTGTCTGAAAGAGCTTTCTGTG-5′ 3′-CCCACCTAGCTGCATTTCAAGTGCTCAAC-5′	65	A: 107 G: 79	*HPY166 II*
TF							
rs12769 (G to A)	p.Ser208Ser	rs12769 F rs12769 R	Sense Antisense	3′-CTCCACCCTTAACCAATACTTCGGCTACCC-5′ 3′-AGGGCTCGGCTTTGGACCAGCAAAGAACTC-5′	50	A:131 G: 99	*Msp I*
TF							
rs1049296 (C to T)	p.Pro589Ser	rs1049296 F rs1049296 R	Sense Antisense	3′-CCATCAGCTGAACCACCTTCTTCCTGTCCC-5′ 3′-TGGCAGTTCGCATACTCCTCCACCG-5′	50	T: 136 C: 110	*Msp I*
TF							
rs1799852 (C to T)	p.Leu247Leu	rs1799852 F rs1799852 R	Sense Antisense	3′-CTGACAGGGACCAGTATGAGCTGCTTGGC-5′ 3′-CACGACGGTATGAGAAGGGACCTGAGCCAA-5′	50	T: 125 C: 94	*Hae III*
TF							
rs3811647 (A to G)	Intron variant	rs3811647 F rs3811647 R	Sense Antisense	3′-CCCTAAGCTGAGGGAGTTTACAGACAGAAC-5′ 3′-ATGTGCAGGCCTCATGTCATGTCAGGTGA-5′	50	A: 105 G: 79	*HpyCH4 IV*
TF							
rs4459901 (T to C)	Intron variant	rs4459901 F rs4459901 R	Sense Antisense	3′-GGGTCTGACCATTCCCCTTTCAGAGGCTGA-5′ 3′-AAGTCTGGCTTCAAACCCCATGCTCTGACC-5′	65	T: 119 C: 88	*HPYCH4 IV*
TFRC							
rs3804141 (A to G)	Intron variant	rs3804141 F rs3804141 R	Sense Antisense	3′-GCCAGGAGTTTAGAGATGGGTAGGTTG-5′ 3′-CCTAAGCTCATAAGAGTCCAGGTGAA-5′	50	A: 137 G: 111	*HPY166 II*
TFRC							
rs3817672 (G to A)	p.Gly142Ser	rs3817672 F rs3817672 R	Sense Antisense	3′-TCGGAGAAACTGGACAGCACAGACTTCACC-5′ 3′-CCCCACAGTGTCACCATTATTGTTTCCCCG-5′	65	A: 130 G: 99	*Msp I*
TFRC							
rs11915082 (G to A)	Upstream variant	rs11915082 F rs11915082 R	Sense Antisense	3′-ACGTACGCTCGCGAGGTGCTCTGACAGAT-5′ 3′-TTGGCGCCTTTTCCCTTGGCCTACG-5′	50	G: 120 A: 94	*HPYCH4 IV*
TFR2							
rs7385804 (A to C)	Intron variant	rs7385804 F rs7385804 R	Sense Antisense	3′-AAAGCCCTGAGCAGGCTGGGTGCGGTGACT-5′ 3′-GTCTCCCTATGTTGCCCAGGATGGTCTCAA-5′	50	A: 121 C: 90	*Hinf I*

Underlined residues indicate the mismatched nucleotide in each primer.

2.2. SNP typing

In this study, polymorphic SNPs in iron homeostasis genes among Japanese postmortem cases were analyzed by polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP) analysis [8,9]. We used a mismatched PCR-amplification method for genotyping because the substitution sites corresponding to these SNPs neither suppress nor create any known restriction enzyme recognition sites. Incorporation of a deliberate mismatch near the 3′-terminus of a PCR primer allowed the creation of each enzyme recognition site. Amplification was performed in a 20-μL reaction mixture using approximately 2 ng of DNA. The reaction mixture contained forward and reverse primers and Green Master Mix (GoTaq, Promega, Madison, WI). PCR was performed with a protocol that consisted of initial denaturation at 94 °C for 3 min followed by 30 cycles with denaturation at 96 °C for 1 min, annealing at 50 °C for 1 min, extension at 72 °C for 2 min, and final extension at 72 °C for 5 min. The PCR product (2 μL) obtained using each pair of primers was digested with the restriction enzyme (Table 1) at 37 °C for 2 h in a final reaction mixture volume of 15 μL, according to the manufacturer's instructions, to determine the genotype of each SNP. The primers and enzymes used for each SNP are shown in Table 1.

The nucleotide sequences of the representative subjects were confirmed by direct sequencing of the PCR products, in which a substitution site corresponding to each SNP was included. The dideoxy chain-terminating method with the BigDye® Terminator Cycle Sequencing Kit was employed using a Genetic Analyzer 310 (Applied Biosystems, Waltham MA) according to the manufacturers' instructions.

2.3. Measurement of blood iron levels

Whole blood samples (0.5 mL) were digested in 2.5 mL of concentrated HNO_3 by heating at 65 °C for 1 h via the hot block acid digestion system DigiPREP Jr (GL Sciences, Tokyo, Japan). After adding 0.5 mL of 30% H_2O_2 to the digest, the mixture was heated at 95 °C for 1 h. The solution was cooled and diluted to 25 mL with ultrapure water. Iron levels in the solutions were determined by a calibration curve ranging from 0.1 to 1 ppm of total iron. Measurements were performed using an Agilent 4200 microwave plasma-atomic emission spectrometer (MP-AES; Agilent Technologies, Santa Clara, CA) equipped with an Inert One Neb nebulizer and a double-pass glass cyclonic spray chamber (Agilent Technologies).

2.4. Statistical analysis

Chi-square analysis was performed to evaluate the Hardy–Weinberg equilibrium. Differences in blood iron concentrations among males and females, among previous studies were compared by the student *t*-test. Univariate (single) regression analysis was used to compare iron concentrations in blood among different genotypes. Differences in the blood iron levels of each genotype were analyzed with Fisher test. Statistical analysis was conducted using the program Statcel2 (OMS Publishing, Inc., Saitama Japan). *P*-values less than 0.05 were considered significant.

3. Results and discussion

In this study, iron concentrations in blood were measured by MP-AES and the results and those from previous studies [10–12] are shown