Original Article

Quantitative Study of Iron Metabolism-related Genes Expression in Rat*

LI Yan Qin[1,#], BAI Bin[2], ZHENG Quan Qing[1], YAN Hong[1], and ZHUANG Gui Hua[1]

1. Department of Public Health, College of Medicine, Xi'an Jiaotong University, Xi'an 710061, Shaanxi, China; 2. Department of Orthopedics, The First Affiliated Hospital of the College of Medicine, Xi'an Jiaotong University, Xi'an 710061, Shaanxi, China

Abstract

Objective To investigate the multiple iron metabolism-related genes expression, its regulation by iron and the expression correlation among the genes in rat tissues.

Methods Two groups (*n*=30) of Sprague-Dawley female weanling rats were fed with a control diet and an iron deficient diet respectively for 4 weeks. All rats were then sacrificed, and blood and tissue samples were collected. The routine blood examination was performed with a veterinary automatic blood cell analyzer. Elemental iron levels in liver, spleen and serum were determined by atomic absorption spectrophotometry. The mRNA expression of genes was detected by real-time fluorescence quantitative PCR.

Results After 4 weeks, the hemoglobin (Hb) level and red blood cell (RBC) count were significantly lower in the iron deficient group compared with those in the control group. The iron levels in liver, spleen and serum in the iron deficient group were significantly lower than those in the control group. In reference to small intestine, the relative expression of each iron-related gene varied in the different tissues. Under the iron deficiency, the expression of these genes changed in a tissue-specific manner. The expression of most of the genes significantly correlated in intestine, spleen and lung, but few correlated in liver, heart and kidney.

Conclusion Findings from our study provides new understandings about the relative expression, regulation by iron and correlation among the mRNA expressions of *transferrin receptors 1* and *2*, *divalent metal transporter 1*, *ferritin*, *iron regulation proteins 1* and *2*, *hereditary hemochromatosis protein*, *hepcidin*, *ferroportin 1* and *hephaestin* in intestine, liver, spleen, kidney, heart, and lung of rat.

Key words: Iron; Tfr; Dmt1; Hepcidin; Ferroportin 1; Hephaestin

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INTRODUCTION

Iron is an essential nutrient involved in many vital processes for life. Both iron deficiency and iron overload are harmful to the health of humans. Therefore, iron homeostasis is tightly regulated normally. The uptake, use, storage and excretion of iron are maintained in a dynamic balance to ensure that the iron content in body can fulfill the physiological needs without excessive accumulation. Factors disturbing iron homeostasis may lead to diseases. Iron metabolism has therefore

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#Correspondence should be addressed to LI Yan Qin. Tel: 86-29-82655108; E-mail: liyanqin@mail.xjtu.edu.cn

Biographical note of the first author: LI Yan Qin, female, born in 1973, Ph.D, associated professor, majoring in molecular epidemiology.

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long been the focus and interest of many researchers.

In the last 10 years, major progress has been made in the field of iron metabolism. Many genes and proteins related to iron metabolism, including *divalent metal transporter 1* (DMT1)[1-2], *transferrin receptor 2* (TfR2)[3], *ferroportin 1* (FPN1)[4-6], *hephaestin* (Heph)[7-9], and *hepcidin*[10-11] have been identified and studied. These and other studies, have greatly improved our understanding about iron metabolism and its regulation, and also provide the scientific basis to the molecular mechanisms of iron metabolism-related diseases. It has been shown that the expression and regulation of these genes is tissue-specific and the expression of these genes may be directly or indirectly affected by the iron status in the body. However, very few studies[12-14] have examined multiple iron metabolism-related genes expressions in multiple tissues at the same time. Consequently, there is limited information on the relative expression level of a specific gene in multiple tissues, whether the expression of a specific gene in several tissues is regulated by iron, and whether the expression levels of multiple iron metabolism-related genes in a specific tissue are correlated. And these gaps in our knowledge are particularly evident for the more recently discovered genes.

We therefore conducted the present study in order to investigate the relative mRNA expression of *transferrin receptors* (*Tfr1* and *Tfr2*), *divalent metal transporter 1* (*Dmt1)*, *ferritin* (*FtH* and *FtL*), *iron regulation proteins* (*Irp1* and *Irp2*), *hepcidin (Hamp)*, *hereditary hemochromatosis protein* (*Hfe*), *ferroportin 1* (*Fpn1)* and *hephaestin (Heph)* in the small intestine, liver, spleen, heart, lung and kidney in rats. We also examined the effects of iron deficiency on the expression of these genes in these tissues, and determined correlations among the expression levels of each gene in each tissue.

MATERIALS AND METHODS

Animals and Diets

Thirty female Sprague-Dawley rats (7 weeks old) were provided by the Laboratory Animal Center of the College of Medicine of Xi'an Jiaotong University [SYXK (Shaan) 2007-003]. Rats [SYXK (Shaan) 2007-001] were kept in cages at a temperature of 17-23 °C and relative humidity of 30%-65% and with a 12-h day/night cycle throughout the study. The rats were first administerd a control diet for 1 week. Then, the rats were randomized into two groups (*n*=15 per group) with one to receive an iron-deficient diet (Fe <8 mg/kg) and the other to continue the control diet (Fe 50 mg/kg) for 4 weeks. The specific details of the diet are as follows: Corn starch 50%; Casein 23%; Fiber 5%; Soybean oil 7%; Vitamin mix 1%; Mineral mix 3.5%; Sucrose 10%; L-Cystine 0.3%; and Choline 0.2%. The difference between iron-deficient diet and the control diet is only the different quantity of $FeSO_4$ in mineral mix. This study was approved by the Medical Research Animal Ethics Committee of Xi'an Jiaotong University.

Blood and Tissue Sampling

Every week, blood samples (20 μL) were collected and tested on a veterinary automatic blood cell analyzer. At the end of Week 4, all rats were anesthetized with 10% hydrated chlorine aldehyde. Ten milliliters of blood was drawn from the heart, collected then in non-heparinized tubes and left overnight at 4 °C. The resulting serum was stored in metal-free Eppendorf tubes at -70 °C for iron element determination afterwards. Small pieces of the full-thickness heart, liver, spleen, kidney, lung and proximal small intestine tissues were dissected with RNAase-free knives, scissors and tweezers. Tissues were rinsed several times with cold DEPC water, snap-frozen in liquid nitrogen and stored at -70 °C in RNAase-free Eppendorf tubes for further analysis.

Atomic Absorption Spectrophotometric Analysis

Samples of the liver and spleen (0.3 g) were subjected to wet digestion for the iron element determination. The iron contents in the liver, spleen and serum were determined with atomic absorption spectrophotometry following standard procedures. Standard curves were prepared by using commercially available standards.

Real-time Fluorescence Quantitative Polymerase Chain Reaction (PCR)

Total RNA was extracted from tissues by using TRIzol reagent (Invitrogen Co., Carlsbad, CA, USA). RNA was quantified with a spectrophotometer (ND-1000, NanoDrop, Wilmington, DE, USA). Sample purity was assessed by calculating the optical density at 260:280 nm and by 1% agarose gel electrophoresis. Total RNA (500 ng) was used for

cDNA synthesis with a RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Burlington, Canada). cDNA was subjected to real-time quantitative PCR in a iCycler IQ5 (BIO-RAD, Hercules, CA, USA) with a SYBR Green detection platform. Primer sequences are presented in Table 1. Although a number of the genes studied have different mRNA isoforms, the primers used in this study did not distinguish the specific isoforms.

PCR was performed in a total reaction volume of 25 μL per tube. The PCR reaction mixture consisted of 12.5 μL of SYBR Green real-time PCR Master Mix (TOYOBO Co., Ltd., Osaka, Japan), 1.0 μL of the forward and reverse primers (10 μmol/L), 9.5 μL of H_2O and 1.0 μL of cDNA. The PCR thermal cycle consisted of denaturation at 95 °C for 60 s, followed by 40 cycles of 95 °C for 15 s, 60 °C for 15 s and 72 °C for 45 s. Melting curve analysis and 2% agarose gel shift assays of the PCR amplification products were used to distinguish interference from primer-dimers and non-specific amplification.

Statistical Analysis

Each sample was normalized for the mean Ct value of β-actin. The relative quantification was performed with the comparative Ct method by using Calculation Software for the Relative Expression in real-time PCR[15], which is based on the calculation of the $2^{-\Delta\Delta Ct}$ value. Correlations between the expression levels of two genes in individual tissues in the control group were analyzed and statistical significance is assumed at $P\leq0.05$.

Table 1. Primer Sequences

Gene	Primers (5'- 3')
Tfr1	F: AGTCATCTGGATTGCCTTCTATACC R: TAGCGGTCTGGTTCCTCATAGC
Tfr2	F: AGAGGAGGAAGATAGGGAGGAAGG R: ACCAACCACCAACACAGAGTCC
Dmt1	F: ATCCTCCTGCCTCTGCCTCTG R: CCTCTCCTGCTCTACCTTTCTTTCC
FtH	F: CAGCGAGGTGGACGAATCTT R: TGACTGATTCACACTCTTTTCCAAGT
FtL	F: GCTGGCTTCTTGATGTCC R: CCTCCTACACCTACCTCTC
Irp1	F: TGATTGATGGTCTGGGAGTTCTTGG R: TTGTCGGAGGTGCTTGGTAATGG
Irp2	F: GCCTGCCAGTTACTCTTACTTTACC R´- TTCCAGCCACGCCTACTTGC
Hamp	F: CTGCCTGTCTCCTGCTTCTCC R´- AGTTGGTGTCTCGCTTCCTTCG
Hfe	F: AAGTTGAGAGTGGTGCCTGAGTC R: CTTGCCCATCGTAGCCATATTTCC
Fpn1	F: GCCACAGTATATGCTTACACTCAGG R: AGAACAGACCAGTCCGAACAAGG
Heph	F: GGTGAAGATGGCAAGCAGAAAGG R: TGGGCAGGTTAGAGAACAGAAATCC
β-actin	F: CTATCGGCAATGAGCGGTTCC R: TGTGTTGGCATAGAGGTCTTTACG

RESULTS

Hb Level, RBC Count, and Liver, Spleen, and Serum Iron Levels

In Week 1, there were no differences in the hemoglobin (Hb) level or RBC count between the two groups (Table 2). However, after 4 weeks, the Hb level and RBC count were significantly lower in the iron-deficient group than those in the control group ($P<0.001$). The liver and spleen of rats in the iron-deficient group were light in color and atomic absorption spectrometry showed that the iron levels in the liver, spleen and serum were significantly lower in the iron-deficient group compared with those on the control group (Table 3).

Relative Expression of Iron Metabolism-related Genes in Individual Tissues

Using the small intestine as a reference, we determined the relative expression of each gene in each tissue (Figure 1). *Tfr1* mRNA expression was highest in the spleen, followed by the intestine, and was lower but similar in the liver, heart, kidney, and

Table 2. RBC Count and Hb Levels in the Two Groups at Week 1 and Week 5

Groups	*n*	RBC (×10^12/L)		Hb (g/L)	
		Week 1	Week 5	Week 1	Week 5
Control	15	7.54±0.39	7.45±0.22	154.07±8.44	151.53±5.17
Iron deficiency	15	7.51±0.17	4.10±0.26	154.26±3.61	86.92±4.11
t		-0.076	-9.953	0.022	-9.891
P value		0.940	0.000	0.983	0.000

Table 3. Elemental Iron Levels in the Spleen, Liver and Serum in the Two Groups at Week 5

Item	Serum (μg/mL)	Liver (μg/g)	Spleen (μg/g)
Control	10.25±0.42	564.23±43.54	2289.13±108.60
Iron deficiency	7.89±0.27	89.09±9.77	205.54±44.04
t	-4.558	-10.647	-17.78
P value	0.001	0.000	0.000

lung. *Tfr2* mRNA expression was only detected in the intestine and liver, and its expression was 5.86-fold higher in the liver than in the intestine. *Dmt1* mRNA expression was highest in the intestine but was lower in the kidney and spleen and, was lowest in the liver, lung and heart. *FtH* mRNA expression was high in the kidney and heart, followed by the intestine, liver and spleen, and was lowest in the lung. Relative to the intestine, *FtL* mRNA was abundantly expressed in the spleen and liver, followed by the kidney, and was lower in the heart and lung. *Irp1* mRNA was highly expressed in the

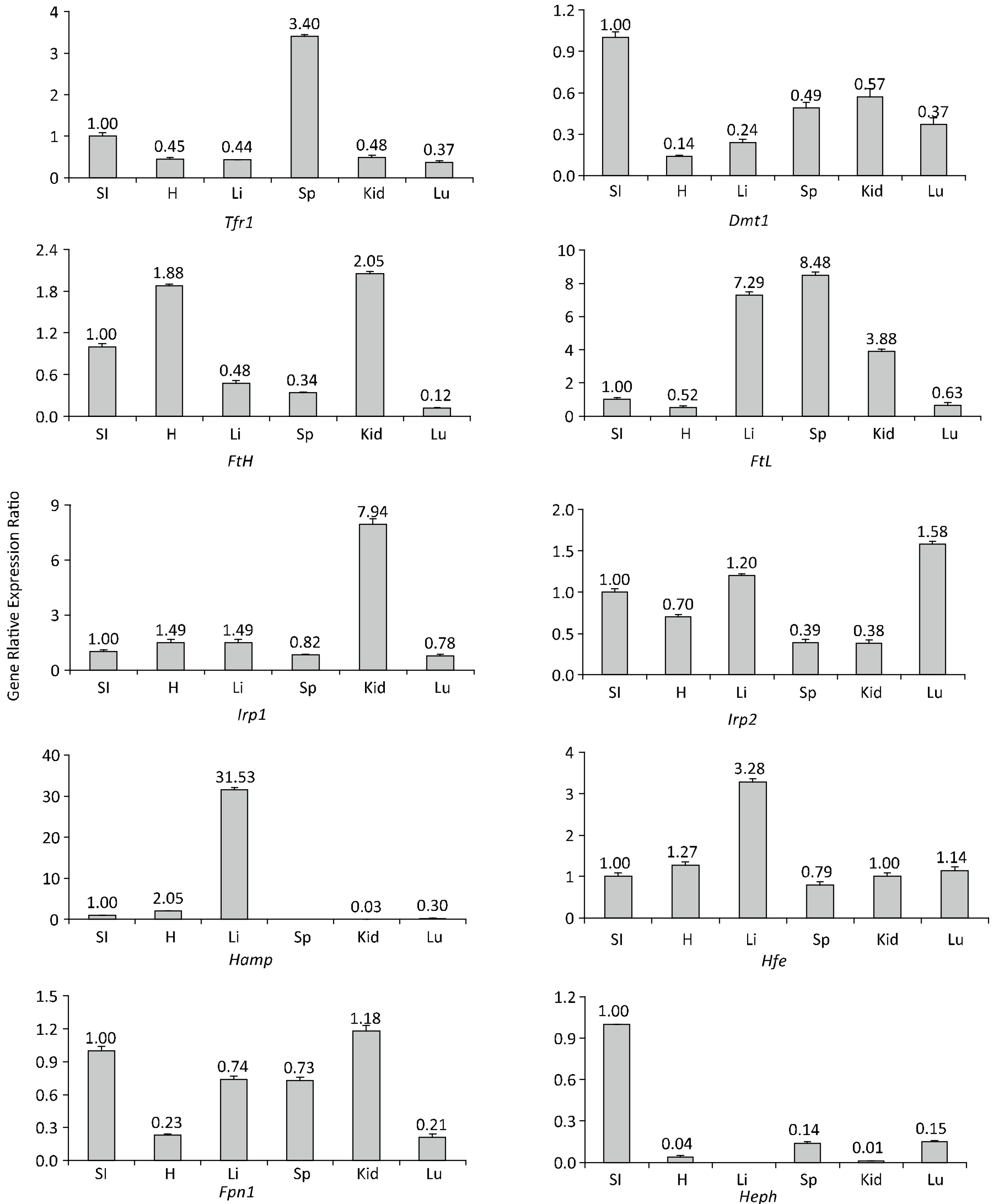

Figure 1. Relative expression of iron-metabolism genes in rat tissues. SI, small intestine; H, heart; Li, liver; Sp, spleen; Kid, kidney; Lu, lung; *Tfr1*, *transferrin receptor1*; *Dmt1*, *divalent metal transporter1*; *FtH*, *H-ferritin*; *FtL*, *L-ferritin*; *Irp1*, *iron responsive protein 1*; *Irp2*, *iron responsive protein 2*; *Hamp*, *hepcidin*; *Hfe, hereditary hemochromatosis protein; Fpn1, ferroportin1*; *Heph*, *hephaestin.*

kidney, and was similar in the other tissues. *Irp2* mRNA expression was similar in the lung, liver, intestine and heart, and was lower in spleen and kidney. *Hfe* mRNA was highly expressed in the liver, and was lower but was similar in other tissues. *Hamp* mRNA was abundantly expressed in the liver and relatively high in the heart and intestine but was very low in the lung and kidney, and was undetectable in the spleen. *Fpn1* mRNA expression was similar in the kidney, intestine, liver and spleen, but was lower in the heart and lung. *Heph* mRNA expression was highest in the intestine, but was very low in the lung and spleen and was negligible in the heart and kidney, and was undetectable in the liver.

Effects of Iron Deficiency on Gene Expression

The effects of iron deficiency on the expression levels of each gene in each tissue are shown in Figure 2. Iron deficiency significantly increased *Tfr1*

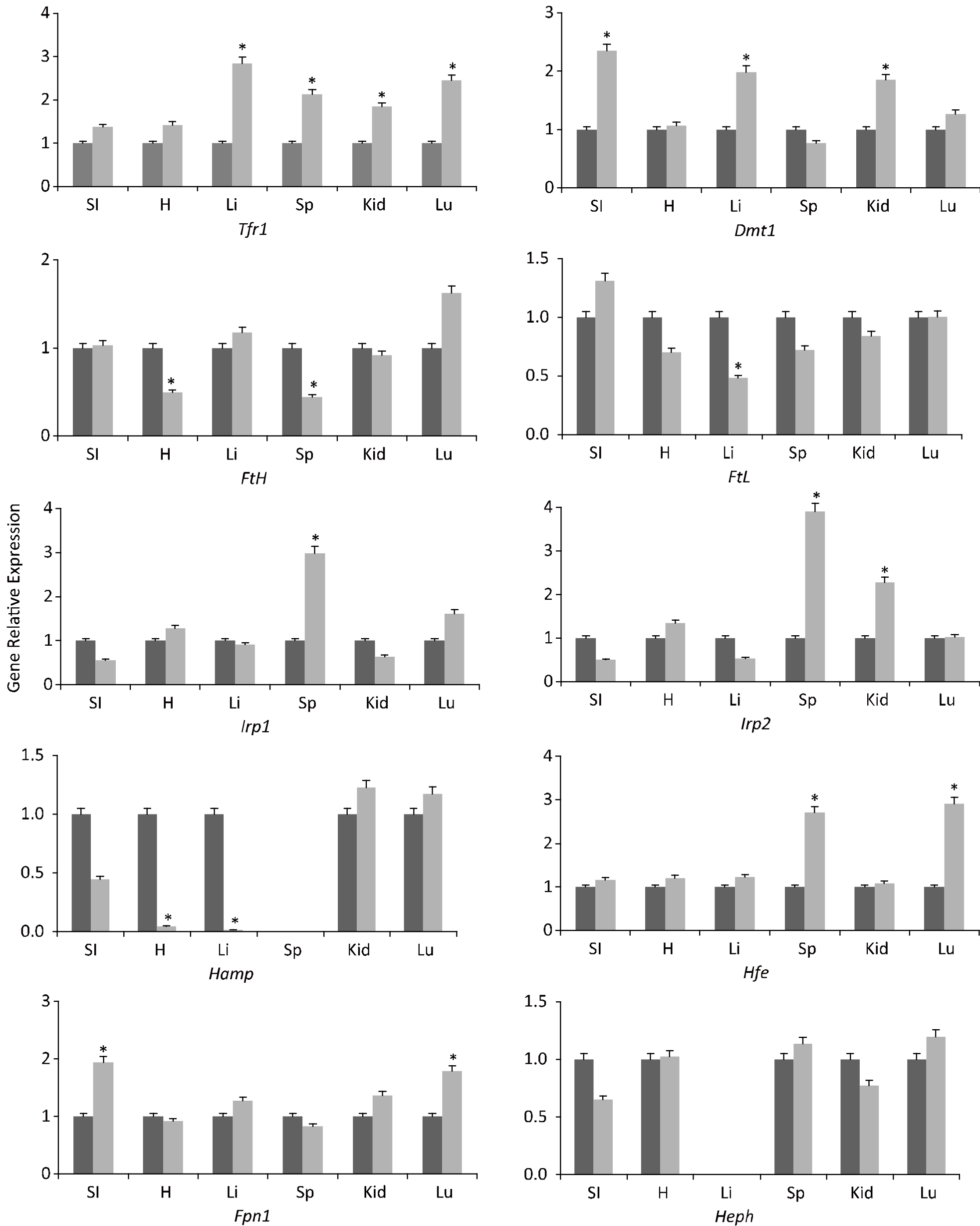

Figure 2. Relative changes in gene expression in various tissues of iron-deficient rats. mRNA expression was determined by real-time fluorescence quantitative PCR. ■control ■iron deficiency, $^{*}P$<0.05 *vs.* control.

mRNA expression in the liver, lung, spleen, and kidney, but not in the heart and intestine. *Tfr2* mRNA expression, which was only detected in the liver and intestine, was not significantly affected by iron deficiency, with 1.23- and 1.03-fold increases, respectively. Iron deficiency significantly increased *Dmt1* mRNA expression in the liver, intestine, and kidney, but not in the heart, lung, or spleen. *FtH* mRNA expression in the heart and spleen, and *FtL* mRNA expression in the liver were significantly decreased by iron deficiency. The mRNA expression levels of *Irp1* and *Irp2* in the spleen, *Irp2* in the kidney, *Hfe* in the lung and spleen, and *Fpn1* in the intestine and lung were significantly increased by iron deficiency while *Hamp* mRNA expression in the liver and heart was significantly decreased by iron deficiency. Still, we found no significant change in the mRNA expression of *Heph* in any tissue.

Correlations among Gene Expression Levels in Each Tissue

In the intestine, the mRNA expression levels of *Dmt1* and *FtL*, *Heph*, *Hfe* and *Tfr2* were significantly correlated. Similarly, there were significant positive correlations between *FtL* and *Hfe*, *Irp2* and *Heph*, and *Heph* and *Hfe* mRNA expression levels (Figure 3). In the spleen, the mRNA expression level of *Dmt1* was significantly and positively correlated with the

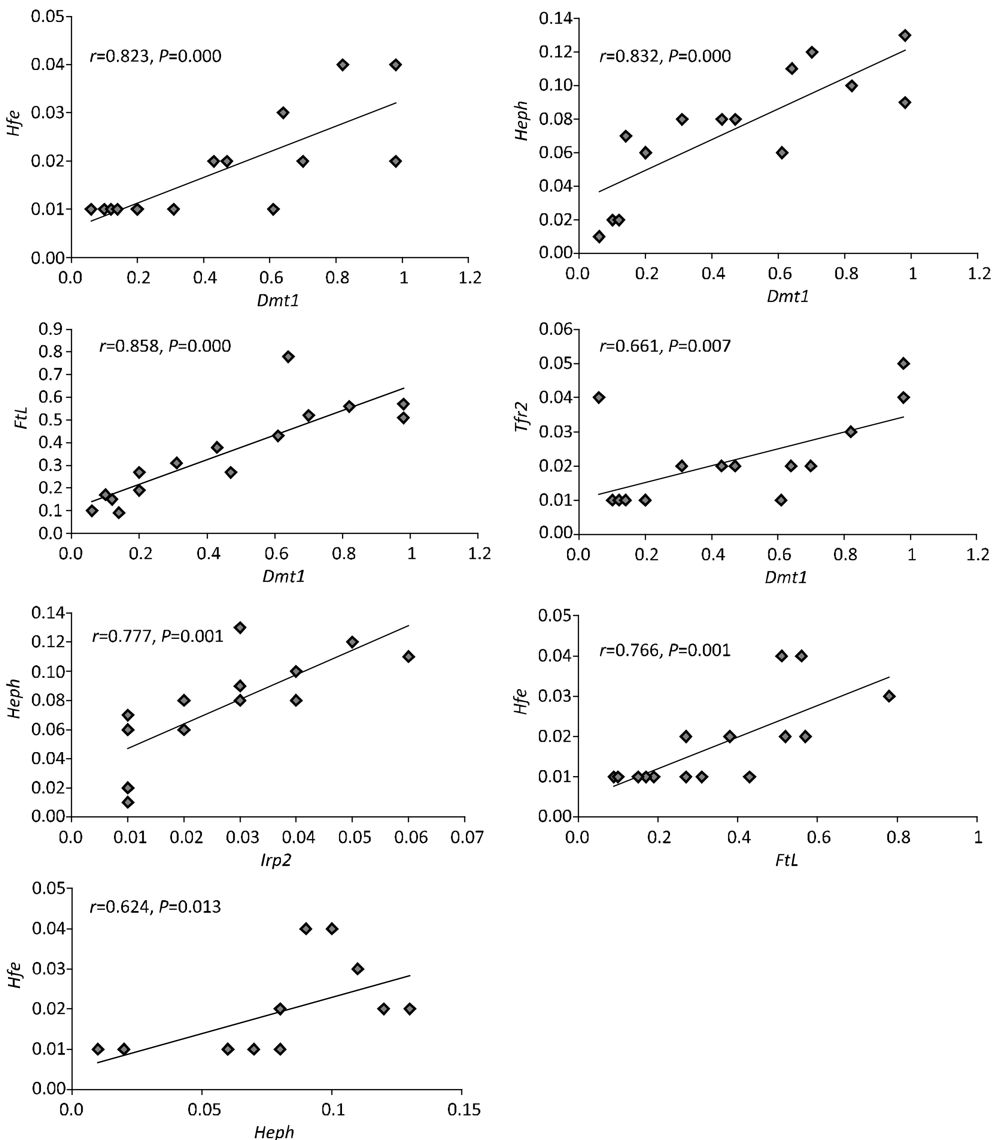

Figure 3. Correlations among the expression levels of iron metabolism-related genes in the intestine. mRNA expression was determined by real-time fluorescence quantitative PCR.

expression levels of *FtH* and *Fpn1*. Significant positive correlations were also observed between *Fpn1* and *FtH*, *FtH* and *FtL*, *Tfr1* and *Heph*, *Hfe* and *Irp1*, *Irp1* and *Heph*, and *Hfe*, *Heph* and *Hfe* (Figure 4). Correlations among gene expression levels in the lung, heart and liver are shown in Figure 5. In the lung, there were significant positive correlations between *Dmt1* and *Fpn1*, *Fpn1* and *FtL*, *Tfr1* and *Irp1*, and *Irp2*, *Irp1* and *Irp2*. In the heart, there was a significant negative correlation between *Tfr1* and *FtL* while there was a significant positive correlation between *FtH* and *Heph*. In the liver, there was a significant positive correlation among *Dmt1* and *FtH*, and *Fpn1* and *FtH*. Surprisingly, in the kidney, there were no significant correlations among the expression levels of any of these genes analyzed.

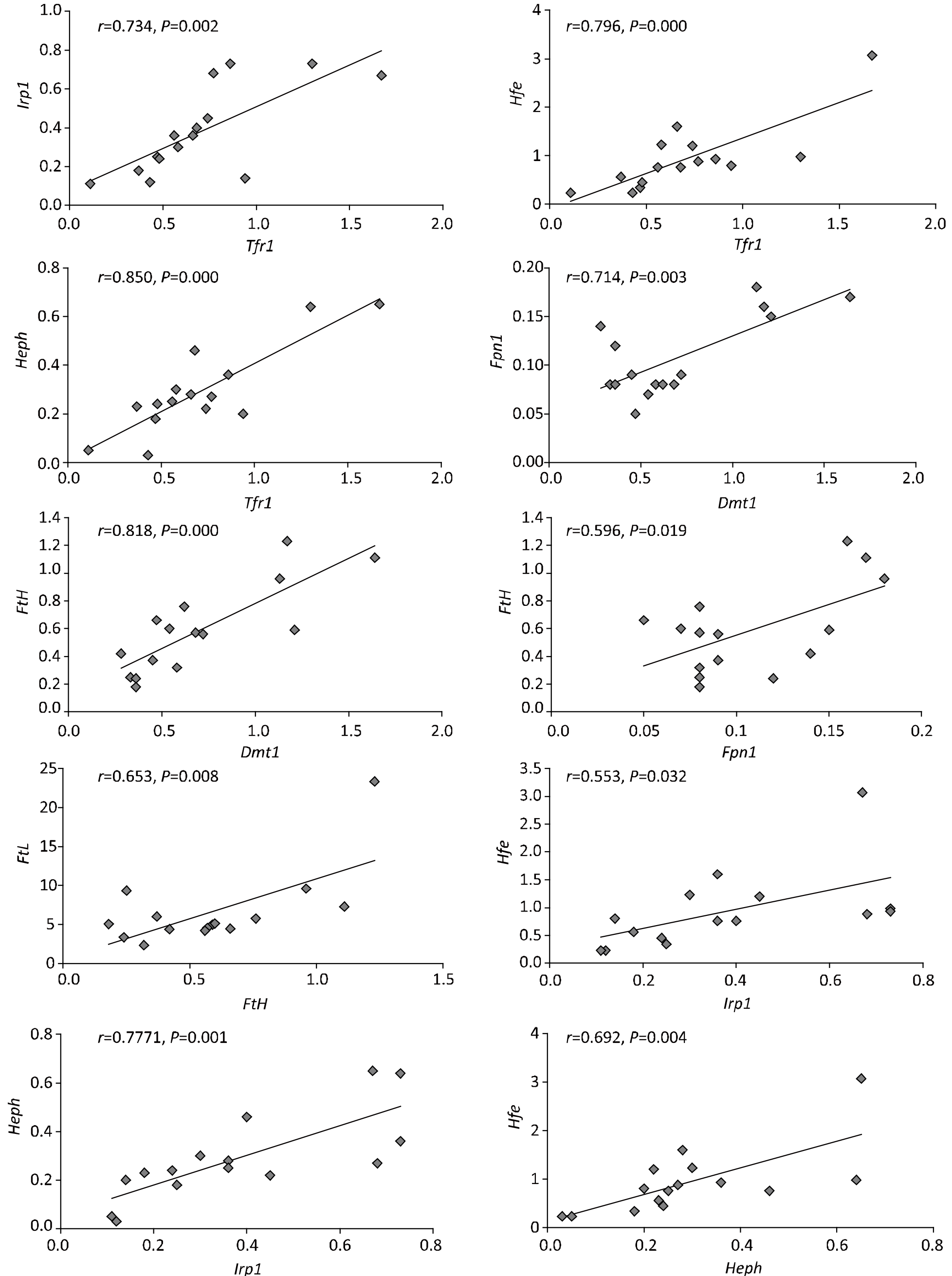

Figure 4. Correlations among the expression levels of iron metabolism-related genes in the spleen. mRNA expression was determined by real-time fluorescence quantitative PCR.

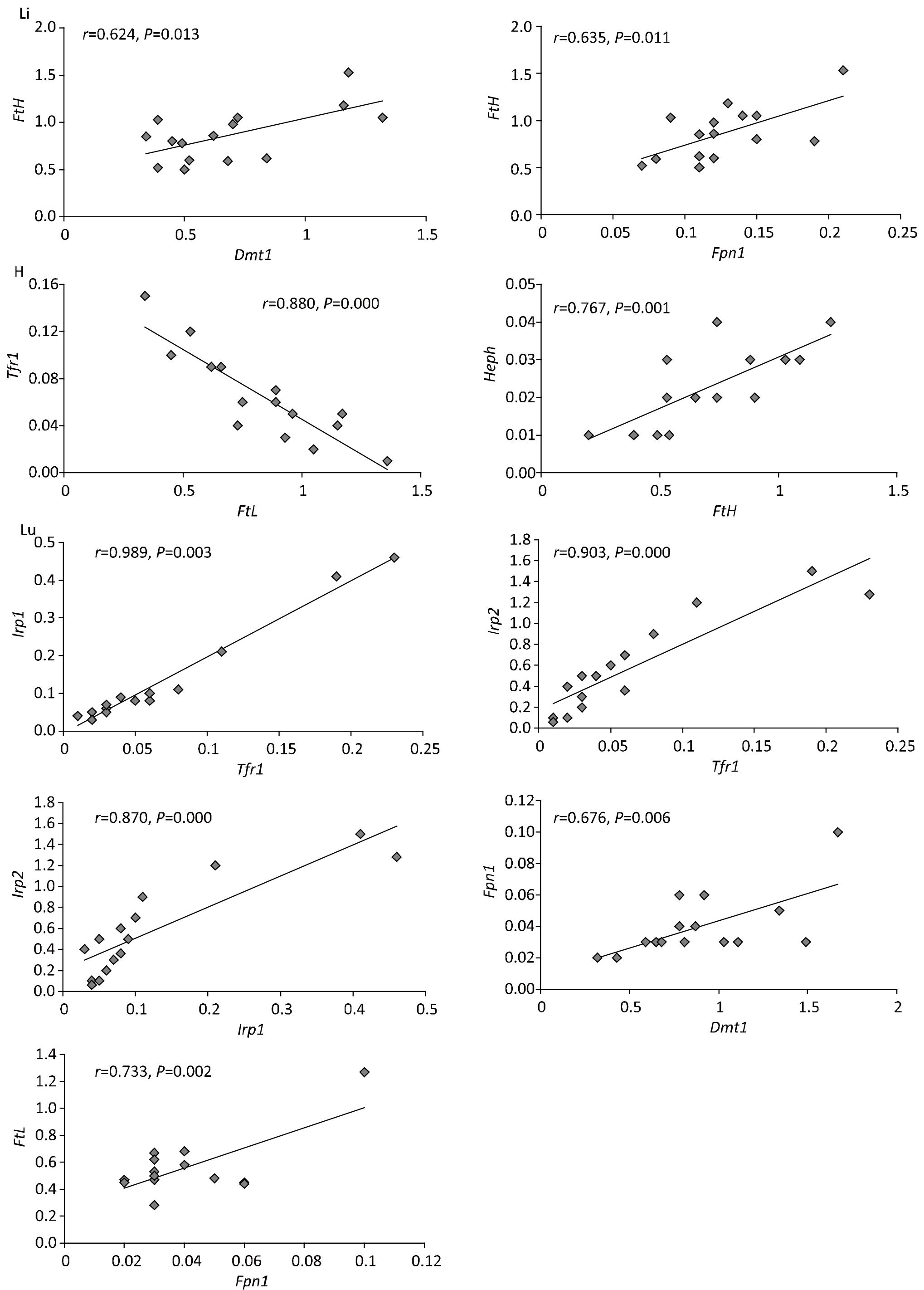

Figure 5. Correlations among the expression levels of iron metabolism-related genes in the liver, heart, and lung. mRNA expression was determined by real-time fluorescence quantitative PCR.

DISCUSSION

Genes Involved in Iron Uptake

Almost all cells obtain at least some of their iron requirements by *TfR1* Tf-mediated iron-uptake[16]. Two types of TfRs, namely *TfR1* and *TfR2,* have been found[17-18]. *TfR1* expression varies considerably among tissues, but is higher in cells with greater iron requirements. *TfR2,* the more recently discovered *TfR,* is similar to *TfR1* in terms of gene and protein

structures, and iron uptake function. However, there are marked differences in the distribution and regulation of these two genes. *Tfr1* mRNA is expressed in almost all tissues and cells, although its expression is lowest in the liver. By contrast, *Tfr2* expression in humans is highest in the liver, and was lower in the stomach and other tissues[19]. In this study, *Tfr1* mRNA expression was higher in the spleen which needs more iron, and was lower in the heart, liver, kidney and lung compared with the intestine. Consistent with other studies, the expression *of Tfr2* was higher in the liver but the expression of *Tfr1* was lower in the liver.

DMT1 is widely distributed in mammalian tissues. *Dmt1* mRNA (4.5 kb) was more strongly expressed in the proximal intestine than in the kidney, and also in the kidney than in the brain. Strong band (3.5 kb) was observed in the kidney and thymus[2]. In the intestine, *Dmt1* expression was highest in the duodenum and the expression decreased towards the colon[2]. Immunoblotting experiments also showed that *Dmt1* was expressed at low levels throughout the small intestine, but at a higher level in the kidney[20]. In this study, we did not distinguish between the *Dmt1* mRNA isoforms. The results showed that *Dmt1* was highly expressed in the proximal intestine and followed by the kidney and spleen, which is consistent with the results from Gunshin et al. (1997).

The protein expression of TfR1 and DMT1+IRE is regulated by iron in most tissues[21-22], although this is not always true for their mRNA expressions[22-25]. Like Gunshin et al. (1997), we found that iron deficiency significantly increased *Tfr1* expression in the liver, lung, spleen and kidney, but not in the intestine and heart. Iron deficiency also increased *Dmt1* expression in the intestine, liver and kidney, but not in the heart, lung and spleen. These results indicate that both of these genes are regulated in a tissue-specific manner. Several studies have shown that the cellular iron content does not directly regulate *Tfr2* expression[21]. And our study provides further evidence that *Tfr2* expression in the liver and intestine is not regulated by iron status.

Genes Involved in Iron Storage

Ferritin molecules are composed of heavy and light chain subunits. The ratio of heavy to light chains can vary depending on the tissue type. *FtL* is predominantly in the liver and spleen and *FtH* is in the heart and kidney[26]. Similar to Arosio et al.[26], we found in this study that *FtL* mRNA expression was higher in the spleen, liver and kidney, while *FtH* expression was higher in the kidney and heart than in the intestine. These differences may be explained by the fact that more iron is stored and used in these organs. Ferritin expression is regulated by a post-transcriptional, iron-dependent mechanism[27]. However, ferritin gene transcription can be modulated by iron-dependent and iron-independent factors[28]. In our study, iron deficiency significantly decreased *FtH* expression in the heart and spleen, and *FtL* expression in the liver. This may be due to that these tissues are the major iron storage tissues, and are potentially more sensitive to changes in iron levels than other tissues.

Genes Involved in the Regulation of Iron Homeostasis

IRPs are RNA binding proteins that bind to the iron regulatory element (IRE) of iron metabolism-related genes and then regulate their mRNA translation or affect their mRNA stability[29]. There are two IRPs, namely IRP1 and IRP2, which are similar but not identical proteins[30]. Because the RNA-binding activity of IRPs plays an important role in iron metabolism, most studies have measured their IRE-binding activity. However, few studies focused on IRPs expression. *In situ* hybridization assays revealed that both IRPs were ubiquitously expressed. But the relative expression levels of the two mRNAs differ in a cell- and tissue-specific manner[31]. *Irp1* mRNA was highly expressed in brown fat, liver, intestine and kidney, and weakly expressed in the spleen and heart. *Irp2* mRNA expression was highest in the adult brain, thymus and retina. The protein levels were consistent with their mRNA levels[32]. Western blot analysis of rat tissue extracts indicated that the protein expression of IRP1 was highest in kidney and liver, while that of IRP2 was highest in the heart and muscle[33]. Another study showed that IRP2 was expressed in all rat tissues, with highest in the intestine, brain and kidney[34]. The results of the present study differ slightly from the previous studies, especially for the *Irp2* mRNA expression and this may come from the different tissues analyzed and methods used. Both IRP1 and IRP2 are regulated by fluctuations in the cellular iron pool, but by different mechanisms. IRP1 is mainly controlled by conformational changes[35], whereas IRP2 is regulated by proteasome-mediated degradation[33]. In this study, iron deficiency caused a significant increase in *Irp1* mRNA expression in the spleen and in *Irp2* mRNA expression in the spleen

and kidney. However, the molecular mechanisms responsible for these changes require further investigation.

HFE is a hereditary hemochromatosis protein. It participates in the regulation of iron homeostasis. The analysis of *Hfe* mRNA expression in different tissues revealed that it was not expressed abundantly in any tissues, but the expression levels were higher in the liver than in other tissues[36]. The results from our study also showed a relatively higher expression of *Hfe* mRNA in the liver, but almost similar expression was found in the other tissues. We also found in our study that iron deficiency increased *Hfe* mRNA expression in the lung and spleen. Yet, the mechanism by which iron regulates *Hfe* expression remains unknown.

Hepcidin is an important and recently discovered regulator of iron homeostasis. *Hamp* mRNA was mainly expressed in liver with weaker expression in the heart, spinal cord and lung, and negligible expression in the prostate, testis, ovary, intestine, kidney, and bladder[37]. The results from our study are basically consistent with the above study. We found the *Hamp* mRNA expression was higher in the intestine than in the lung. *Hamp* expression is regulated by iron status, infection and inflammation[38]. Consistent with other studies, we found that *Hamp* expression in the heart and liver was significantly decreased by iron deficiency.

Genes Related to Iron Export

FPN1 is widely distributed throughout the body, and *Fpn1* mRNA is highly expressed in the adrenal, spleen, liver and placenta[39-40]. In the present study, *Fpn1* mRNA expression was relatively higher in the kidney, intestine, liver and spleen, but lower in the heart and lung, which is basically consistent with those previous studies. Despite the presence of an IRE in *Fpn1* mRNA, its expression is not completely dependent on the IRE/IRP mechanism. Aside from the two major regulatory mechanisms involving IRE/IRPs in the liver and lung and Hepcidin in the intestine[41-42], the expression of FPN1 is also regulated by developmental and post-transcriptional mechanisms[43]. In this study, we found no changes in its expression in tissues other than the intestine and lung in response to iron deficiency. And this may be due to the fact that FPN1 is predominantly regulated at the post-transcriptional level.

Heph is a putative multi-copper oxidase. The main function of Heph is to mediate iron transport in coordination with FPN1. *Heph* mRNA expression was highest in the colon, rectum and jejunum, but barely detectable in the spleen, liver, and placenta[9]. In this study, *Heph* expression was highest in the intestine, very low in the lung and spleen, negligible in the heart and kidney, and undetectable in the liver. These results are similar with some previous studies mentioned above. Heph expression can be regulated by iron, copper and zinc[44-46]. Yet, Heph is not regulated by the IRE/IRP mechanism because there is no IRE in its mRNA. It was reported on the other hand that post-translational modification of Heph may play a significant role in regulating its expression[44]. And in our study, *Heph* expression was unaffected by iron deficiency in any of the tissues tested, which differs from a previous study showing that *Heph* expression was up-regulated in the small intestine of iron-deficient rats[47].

Regarding the correlation analysis, it was revealed that the expression levels of most iron metabolism-related genes were significantly correlated in the intestine, spleen and lung, whereas few genes were correlated in the liver, heart and kidney.

In short, this study provides relatively more comprehensive and comparative information on mRNA expression of the multiple iron metabolism-related genes in various tissues of rats. Yet, further deduction can not be made since the expression of these genes at the protein level was not detected. The other limitation of this work is that some of the genes studied have different mRNA isoforms, yet these are not analyzed separately because the primers used in the study picked up total mRNA and did not favor any specific isoform. We believe that further studies are needed to clarify the mechanism of the mRNA expression changes of these iron metabolism-related genes in various tissues of rats.

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