## Article Title: A Gene-Based Recessive Diplotype Exome Scan Discovers *FGF6*, a Novel Hepcidin-Regulating Iron Metabolism Gene

Short Title: *FGF6* in Iron Metabolism

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## Abstract

Standard analyses applied to genome-wide association data are well-designed to detect additive effects of moderate strength. However, the power for standard GWAS analyses to identify effects from recessive diplotypes is not typically high. We proposed and conducted a gene-based compound heterozygosity test to reveal additional genes underlying complex diseases. With this approach applied to iron overload, a strong association signal was identified between the fibroblast growth factor-encoding gene, *FGF6,* and hemochromatosis in the central Wisconsin population. Functional validation showed FGF-6 regulates iron homeostasis and induces transcriptional regulation of hepcidin. Moreover, specific identified *FGF6* variants differentially impact iron metabolism. In addition, FGF6 downregulation correlated with iron metabolism dysfunction in systemic sclerosis and cancer cells. Using the recessive diplotype approach revealed a novel susceptibility hemochromatosis gene and has extended our understanding of the mechanisms involved in iron metabolism.

**Key Points**

* An exome scan for recessive effects reveals *FGF6* as a hemochromatosis susceptibility gene.
* FGF-6 decreases ferrous iron uptake in liver cells and induces increased hepcidin expression.

**Introduction**

Genome-wide Association Studies (GWAS) are well-designed to detect additive effects of modest effect sizes. We hypothesized that gene-based tests sensitive to recessive diplotypes—including recessive single site effects and compound heterozygosity—may reveal additional genes underlying complex diseases. Carrying variants conferring a compromised function on both homologous chromosomes is likely to impact molecular physiological states. Deep sequencing studies have conclusively shown a vast reservoir of rare variants segregating in human populations.1 Rare variants in functional categories (e.g., missense, regulatory motifs) may generate pathogenic effects through recessively-acting diplotypes, and such effects are apt to remain concealed from standard GWAS analyses. Simple power calculations show that recessive diplotype inheritance produces signals that are difficult for standard GWAS methods to discover (**Supplementary Figure 1**). Compound heterozygosity disease models also enjoy a high degree of biological plausibility, particularly if the alleles confer compromised protein function.2-5 Recessive diplotype modes of inheritance are well-established in Mendelian diseases, such as cystic fibrosis6, mevalonic aciduria7, beta-thalassemia8 and Niemann-Pick disease.9 Although not systematically examined in population-based studies, there is a sizable repository of genes underlying complex diseases with recessive, loss-of-function effects.10-14 Hence, we posited that an exome-wide, gene-based screen of recessive diplotypes using putative functional variants in both oligogenic and complex diseases may expand our knowledge of disease genes.

Iron metabolism disorders, including adult hereditary hemochromatosis, collectively are common conditions with considerable public health implications.15,16 Importantly, the hepatic hormone hepcidin is a key regulator of iron homeostasis by controlling iron flux from enterocytes and macrophages to plasma through degradation of the cellular iron exporter ferroportin (*SLC40A1*). Within cells, ferritin is the iron-storage protein which can be used for indirect iron quantification. To investigate the inheritance of hemochromatosis, several segregation analyses were initially conducted, concluding that a recessive mode of inheritance is highly plausible.17,18 Several studies in humans have investigated the genetics of iron overload, revealing several critically important genes. Notably, *HFE*, encoding the membrane-bound hereditary hemochromatosis protein, was mapped two decades ago through family-based linkage19-22 and association approaches.23,24 Additional studies have definitively placed the missense polymorphism C282Y (rs1800562) in *HFE* as the major susceptibility factor in adult-onset, type 1 hereditary hemochromatosis.25,26 Additional genes have been identified through pathway-based genetic association studies and GWAS, including *BMP2, BMP4, HJV, TF, TMPRSS6, NAT2, FADS2*, and *TFR2*.27-29

#### Methods

#### Central Wisconsin Hemochromatosis Sample Set

The homogenous population in rural Central Wisconsin is the source population for the Personalized Medicine Research Project (PMRP), a biobank linked to electronic health records (EHRs) housed by the Marshfield Clinic Research Institute.30 Samples from over 20,000 individuals comprise the PMRP. The study was conducted in accordance with the Declaration of Helsinki. All samples were collected following written informed consent. Marshfield Clinic received a Certificate of Confidentiality from the National Institutes of Health. All investigators using the PMRP samples had obtained Research Ethics and Compliance Training certification through the CITI program. The study protocol was reviewed and approved by the Marshfield Clinic Institutional Review Board (details in **Acknowledgements**). The Central Wisconsin population is largely stationary and primarily derived from Bavarian migrants in the late 1800s. The population carries high utility for disease gene mapping through reduction in confounding by population stratification and lower expected levels of allelic and locus heterogeneity. In addition, environmental exposures are thought to be relatively uniform across this population. For these reasons, the PMRP has been effectively used in numerous human genetics studies.31-34 PMRP DNA samples were collected and stored approximately 14 years ago and all individuals have longitudinal EHR information housed at the Marshfield Clinic, averaging in excess of 30 years. The EHR is composed of ICD-9 diagnostic codes, laboratory test results, clinical procedure data, prescription information and physician notes. Hemochromatosis cases and controls were selected from the PMRP population. PMRP individuals were selected as hemochromatosis cases on the basis of percent transferrin saturation laboratory values (the ratio of serum iron to transferrin iron-binding capacity) exceeding 48% and having two or more instances of ICD-9 codes indicating the diagnosis of hemochromatosis: 275.0 (iron metabolism disorder, excluding anemia), 275.01 (hereditary hemochromatosis), 275.03 (unspecified hemochromatosis), and/or 275.09 (other iron metabolism disorders). To reduce confounding by population stratification, a Principal Components Analysis (PCA) on the exome genotyping data was implemented using all samples, blinded to disease status. Individuals considered genetic background outliers (more than three standard deviations from the centroid of the first two principal components) were excluded from the study. Following the removal of outliers, the resulting set of individuals was highly homogeneous based on the first three principal components. Exhaustive pairwise kinship coefficients were calculated and one individual from pairs of individuals exhibiting third-degree or closer relatedness were removed. Of the approximately 10,000 individuals previously subjected to the exome genotyping array and quality control procedures, the phenotype algorithm identified 18 individuals that were selected as hemochromatosis cases. Controls (n=6,896) were individuals without abnormal saturation values and without any instances of hemochromatosis ICD-9 codes.

#### Genotyping

Of the full PMRP cohort, approximately 10,000 DNAs were interrogated by high density genotyping on the Illumina HumanCoreExome beadchip. The exome array has ancestry informative markers, a panel of identity-by-descent SNPs, coverage of markers found to be genome-wide significant in GWAS studies, and excellent coverage of exonic variants. Over 500,000 variants are interrogated on this genotyping beadchip. The version of the beadchip was designed and used in the AMD consortium.34 Rare variants (<1% frequency) represented 47.8% of the markers, moderately common variants (1-10% frequency) were 8.1% of the variants, and 44.1% of the variants interrogated were common alleles (>10% frequency). The genotyping quality control measures were previously described (call rates for each variant or individual >0.985).34 Variants exhibiting departure from Hardy-Weinberg equilibrium (p<1x10-6) were excluded from subsequent analyses. Additional recent studies have used data generated from this genotyping platform to discover susceptibility genes for common diseases.35 Following QC procedures, 413,701 variants remained for analysis. The site frequency spectrum of the resulting variants is displayed in **Supplemental Figure 2**.

#### Haplotype Phasing

In general, gametic phasing is necessary to directly determine compound heterozygous individuals at a particular gene. Using all 10,000 exome-genotyped samples from the PMRP, the software package Beagle was applied to infer phased haplotypes from the unphased genotype data using a localized haplotype-cluster model algorithm.36 The calculations were performed on a high performance computing cluster housed at the Marshfield Clinic. As the subsequent analyses were gene-based and the genotyping data was concentrated on exonic variants, each gene in the exome was phased separately using this approach. Although rare variants can present difficulties in phasing, the use of a large sample size from a highly homogeneous population aids in mitigating the error rate. Notably, Beagle has been shown to have error rates in phasing between 0.77-0.94% for medium (n=1,000) to large (n=5,000) sample sizes using a 500K GWAS array.36 Recently, the switch error rate was calculated for the Beagle software applied to two sequencing datasets. Beagle attained a switch error rate of 1.525% and 0.488% for the 1000 Genomes Project and Haplotype Reference Consortium, respectively.37 Whereas phasing accuracy is generally compromised with rare variants, for situations where an individual has substantial number of pathogenic variants, it should be noted that randomly assigning alleles to a homolog in individuals carrying multiple putative functional alleles at a gene yields a probability that the individual has a recessive diplotype configuration of , where is the number of putative functional variants. Hence, an individual carrying four, six or eight putative functional variants at a gene would have a probability of carrying a recessive diplotype of 0.875, 0.969 and 0.992, respectively, given random assignment of alleles to homologs.

#### Determination of Putative Functional Variants

Following the phasing of the genotype data, putative functional variants were identified. The putative functional variants included in the analyses satisfied the quality control criteria as described previously.34 Markers used in the analyses were either GWAS-significant as of June 2015 and/or annotated as missense, nonsense, 3’UTR, 5’UTR or occurring within a splice site region. Annotation was performed using the ANNOVAR software.38 Following filtering for putative functional variants, 129,556 SNPs remained for use in our gene-based recessive diplotypes scan.

#### Statistical Tests of Recessive Diplotypes

At each gene, individuals were classified as having a recessive diplotype configuration if they carried at least one putative functional allele on each homolog (). Individuals carrying at least one homolog free from putative functional alleles were deemed as having a wildtype diplotype (). The total number of case and control individuals carrying a recessive diplotype was denoted by and , respectively. Similarly, the total number of case and control individuals carrying a wildtype diplotype was denoted by and , respectively. Following the determination of these counts, a Fisher’s exact test was applied to the contingency table. As the hypergeometric null density holds for all sample sizes, the Fisher’s exact test is robust to imbalance between case and control sample sizes. Simulations have recapitulated this finding showing that the Fisher’s exact test does not inflate type I error rates under unbalanced designs.39 To investigate the sex-specific effects, the Haldane’s OR was Individuals carrying one or more homozygous genotype(s) at a single site for a putative functional allele were included in the category. Genes without any high quality, putative functional alleles across all samples were removed from the analyses. Across all genes with analyzable data, a conservative experiment-wise correction was used to correct for multiplicity using 15,900 gene-based tests. To compare the recessive diplotype analysis procedure to a standard rare variant gene-based test, the RVTESTS software40 which implements the sequence kernel association test was also applied to the genotype data.41 Additionally, to investigate the sex-specific effects, the Haldane OR was calculated separately for female and male strata. The Mantel-Haenszel joint OR was then calculated to obtain an estimate of effect sizes conditioned on the sex variable.42 Lastly, the Mantel-Haenszel test of homogeneity was calculated to determine the level of statistical evidence for sex-specific differences in effects.42

#### Power Calculations

To explore the efficacy of the approach proposed here, we performed analytic power calculations under the alternative model of compound heterozygosity/recessive inheritance of disease at two sites, each segregating two alleles. By doing so, we sought to compare the power of a standard GWAS analysis (Armitage trend test) to a log-likelihood ratio test.42 **Supplemental Figure 1** shows the power of each of these tests across different sets of penetrances and haplotype frequencies. To consolidate the different sets of haplotype frequencies, the results are plotted as a function of linkage disequilibrium between the two sites. The power of the test for recessive diplotypes exceeded the power for the Armitage trend test across virtually all of the parameter space. Additional work in this area was recently performed by Sanjak and colleagues showing similar results.43

#### Comparative genomic analysis and Protein-protein interaction inference

Amino acid sequencing of the core iron metabolism genes were collected including *TFRC, FTH1, IREB1, SKP1, SKP1, ACO1, TFR2, TF, HMOX1, ACO2, HAMP, FGF6, and FGFR1*. The alignments were derived from NCBI BLASTn database. Phylogeny for different genes were compared to show the earliest evolutionary time point and then occurrence for each gene were mapped to the phylogenetic tree.44 Protein-protein interaction network inference was conducted to FGF-6 and main iron metabolism proteins. The finial network was tuned after removing non-necessary nodes between FGF-6 and key iron molecules including HFE and SLC40A1.

#### Cell culture, Reagents, and Protein treatment

Colon cancer cell lines (HCT-8 and HCT116), a kidney cancer cell line (786-O), a liver cancer cell line (HepG2) and a fibroblast cell line (HFF-1) were cultured in DMEM medium supplemented with 10% FBS at 37°C in a 5% CO2 humidified incubator. **We apply Ferric ammonium citrate (FAC) cell culture method to investigate the iron uptake changes under different protein treatment or plasmid transfection in which cells were cultured in normal DMEM and FBS medium with the presence of 1uM Ferric ammonium citrate (FAC) and 50uM ascorbate for 48h when detecting cellular iron concentration. Total cell iron contents in cells cultured with FAC for 48 hours were much higher than cells with normal cultured cells which is quite low almost no any irons were detected.** Recombinant human FGF-6 protein (Active) (ab219122), anti-Ferritin (ab75973) were purchased from Abcam, Flag tag antibody (20543-1-AP) was purchased from Proteintech Group, anti-GAPDH antibody was purchased from Shanghai Yeasen Biotechnology. 1mM Ferric ammonium citrate and 50mM ascorbate were dissolved in distilled water. NaOH, HCl, KMnO4, ferrozine, neocuproine, ammonium acetate, ascorbic acid and FeCl3 were purchased from Beijing Oka Biological Technology. Plasmid with raw FGF6 sequence was purchased from PPL-Shanghai Co., Ltd (Shanghai, China) which was constructed in an N-Terminal p3XFLAG-CMV vector, whereas three different FGF6 mutations (E127X, D174V, R188Q) were synthesized with overlapping-PCR.

**Quantification of Iron content by ferrozine assay**

Total intracellular iron content was measured by the ferrozine assay.45 Cells were cultured in 12-well plate for 48h and washed three times with cold PBS. After lysed 2h with 50mM NaOH, 100μL of cell lysates were mixed with 10mM HCl, and 100μL of the iron-releasing reagent (a freshly mixed solution of equal volumes of 1.4M HCl and 4.5% (w/v) KMnO4 in H2O). The mixtures were incubated for 2h and 30μL iron detection reagent (6.5 mM ferrozine, 6.5 mM neocuproine, 2.5M ammonium acetate, and 1M ascorbic acid) was added, after 30min incubation, 280μL of solution was added to a 96-well plate and read 550nm on a microplate reader. In addition, FeCl3 (0-100μM) was used as iron standards and protein quantification was determined by a Lowry protein assay.

**Western blot**

Cell lysates were harvested when incubated iron for 48h, then equal amounts of protein from every sample were subjected to 12% SDS-PAGE gels electrophoresis and then transferred to PVDF membranes. After blocked with 5% BSA, the membranes were incubated with GAPDH (1:10000), Ferritin (1:1000) and Flag (1:2000) at 4°C overnight. Then, membranes were washed three times with TBST, incubated with anti-rabbit or anti-mouse secondary antibody. The bands were visualized using Image QuantTL software.

**Perls’ staining**

Cells were washed with phosphate-buffered saline (PBS) three times, fixed with 4% glutaraldehyde for 10 min, and incubated at 37°C for 60 min with 2 ml Prussian blue solution comprising equal volumes of 2% hydrochloric acid aqueous solution and 2% potassium ferrocyanide (II) trihydrate. After the cells were stained with 0.5% neutral red for 3 min, iron staining was visualized by Nikon microscope. Iron positive high positive staining cells divided by total cell number used to evaluate the iron deposition levels.

#### RT-PCR and quantitative RT-PCR analysis

Total RNA was extracted from the cells using Trizol (Invitrogen, Carlsbad, CA, USA). One microgram of total RNA was subjected to cDNA synthesis using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer’s instructions. The specific primers for each gene were designed using Primer 5 and synthesized by Generay Biotech Co., Ltd. (Shanghai, China). The RT-PCR amplification was conducted using a SYBR Green I PCR Kit (TaKaRa, Shanghai, Japan) according to manufacturer’s instructions. The reaction was carried out on a ABI Prism 7900 Detector System (Applied Biosystems). RT-PCR conditions were 95°C for 3 min, followed by 40 cycles of 95°C for 15 s, 60°C for 40 s, and the conditions for obtaining the dissociation curve were 95°C for 15 s, 60°C for 15 s, 95°C for 15 s. The data obtained from the assays were analyzed with SDS 2.3 software (Applied Biosystems). For each sample, the relative gene expression was calculated using a relative ratio to GAPDH. Related RT-PCR primers can be found in the **Supplementary Table 2**.

#### Immunohistochemical staining of FGF-6

The primary antibody used was anti-FGF-6 (1:200, D162668 BBI, Shanghai). Liver and skin tissues from four liver cancer patients and six SSc patients, respectively, and normal controls were formalin-fixed and paraffin-embedded. Sections were deparaffinized and incubated with 5% bovine serum albumin for 60 min. Cells positive for FGF-6 were detected by incubation with the primary antibody for 2 h at room temperature followed by incubation with 3% hydrogen peroxide for 10 min. Rabbit anti-rabbit lgG labeled with horseradish peroxidase were used as secondary antibodies. The expression of FGF-6 was visualized with 3,3-diaminobenzidinetetrahydrochloride (DAB-4HCl). The expression of FGF-6 in SSc and tumor tissues was quantitated by the average optical density (AOD) of positive signal in each sample using the software imageJ (Windows and Java-1.8.0, NIH).

**Results**

#### Gene-based compound heterozygosity identified novel hemochromatosis susceptibility gene

To discover novel iron overload-predisposing genes, we conducted a gene-based scan for recessive diplotypes composed of putative functional alleles across the exome using biobanked samples linked to electronic medical records obtained from a rural, genetically-homogeneous population in central Wisconsin. Of the 10,000 samples evaluated, our transferrin saturation and diagnostic code-based phenotype algorithm identified 18 case individuals and 6896 controls. We estimated gametic phase on all individuals and restricted our analyses of diplotypes to putative functional variants. Our recessive diplotype scan identified two exome-wide significant genes (**Figure 1, Table 1 and Figure S2**), *HFE* (*P=*1.29×10-8; OR=28.7) and *FGF6 (P=1.99*×10-6; OR=22.8*)*. For comparison, the SKAT/rvtest procedure on the *FGF6* genotype data yielded an asymptotic *P=3.86*×10-5 and permuted *P=1.0*×10-4. We found no statistical evidence of effect differences between females and males for the *FGF6* data (Mantel-Haenszel test of homogeneity *P*=0.728). These results motivated our investigation of FGF-6 function and the impact of specific *FGF6* variants on iron metabolism.

#### Comparative genomic analysis reveals FGF6 evolved synchronously with iron metabolism genes

To explore the involvement of *FGF6* in iron metabolism, we found evidence for FGF-6 interactions with FGFR1, MAPK1/3, INS, FN1 and involvement in the iron metabolism subnetwork involving TF, HFE, HAMP and SLC40A1 (**Figure S3**) by investigating FGF-6 protein-protein interactions. FGF-6, also known as Heparin Secretory-Transforming Protein 2 or Heparin-Binding Growth Factor 6 has multiple heparin binding sites (HBS). Three known nonsynomymous variants located in the heparin binding sites (R188Q) or flanking sites (D174V and E172X) were speculated to be important for the FGF-6 function. Further, D174V and E172X are located in the regions between FGFR-binding region (FGFR-BR-3) and HBS-1 (**Figure 2**). Hence, we studied these three variants in functional studies to further investigate the involvement of FGF-6 in iron metabolism.

**FGF-6 modulation of hepcidin expression and iron uptake**

To investigate the potential mechanism linking FGF-6 to iron metabolism, the effects of FGF6 on iron uptake and the expression of iron-metabolism genes in HepG2, HCT8, HCT116, 786-O and HFF1 cells were evaluated. Using cells cultured cells and a ferrozine assay to detect iron, total intracellular iron concentration was significantly decreased in HepG2, 786-O, HCT8, HCT116 and HFF-1 cells when treating with active FGF-6 protein in a dose-dependent manner (**Figure 3A-D and Fig. S4**). Testing the effect of FGF6 on the expression of a set of genes involved in iron metabolism (*HAMP*, *HDAC2*, *HMOX1*, *TFRC*, and *HEPH*), HepG2 cells were subjected to treatment from control or FGF-6 protein and FGF6 mRNA or control and mRNA expression relative to *GAPDH* was measured in the five iron metabolism genes. RT-PCR analysis revealed that *HAMP* and *HDAC2* mRNA levels were significantly increased after the FGF-6 active protein introduction in HepG2 cells compared to treatment with PBS as control (**Figure 4A**). *FGF6* plasmid transfection significantly increased *HAMP*, *HDAC2*, and *HMOX1* levels, whereas *TFRC* levels significantly decreased in HepG2 compared to a vector without *FGF6* (**Figures 4B**). In order to investigate the effects of the *FGF6* alleles on FGF-6 function, we transfected plasmids carrying either the wildtype *FGF6* or variant *FGF6* with each of the three point mutations described above (**Figure 2 and Fig S5**). The M1 (E172X) and M3 (R188Q) variants exhibited a downregulation of HAMP compared to WT in HepG2 cells (**Figure 4C**). Further, TFRC expression was significantly upregulated in the presence of M3 compared to WT (**Figure 4C**). For HCT-116 cells, both M1 and M3 variants significantly reduced the expression of HAMP compared to the introduction of WT *FGF6* (**Figure 4D**). Examining the impact of the specific variants on intracellular iron concentration, M1 and M3 produced elevated iron deposition **(Figure 4E, 4F, Fig. S6B)** and ferritin expression (**Figure 4G-H, Fig. S6C-D**) indicating a deficiency in variant FGF-6-mediated iron uptake-suppression. In addition, the intracellular iron accumulation was confirmed by IHC using Perls’ stain **(Figure S5)**. *HEPH* expression did not change, suggesting the effect of FGF-6 may be independent of *HEPH*. Further, we noted *HAMP* mRNA levels in M1 and M3 transfections were comparable to control levels, which illustrated a strong attenuation of *FGF-6* function for M1 and M3 variants (**Figure 4C-D, Figure S6A**). The M2 variant (D174V) significantly differed from wildtype in *HAMP* induction only in HepG2 cells. (different variants have different affect to different organs)

#### Altered FGF6 gene expression in systemic sclerosis and cancer

We hypothesized that FGF-6 might be involved in human autoimmune diseasesandcancers since abnormal iron metabolism has been reported in numerous studies.47-50 More specifically, decreased hepcidin has been implicated in the anemia of chronic disease which frequently accompanies these systemic inflammatory states. To explore the relationship between *FGF6* expression and iron deposition in autoimmune tissues, *FGF6* expression and iron deposition in the skin lesions from systemic sclerosis patients (SSc) and healthy controls were examined. We found significantly decreased FGF-6 protein by immunohistochemistry assay (**Figure 5A**) and elevated iron deposition in SSc skin tissue by Ferrozine assay (**Figure 5B**), especially in the epidermis. Increased iron deposition was confirmed by Perl’s stain in SSc skin tissues (**Figure S8A**). We also investigated the relationship between FGF6 protein expression with iron deposition in liver cancer tissues. We found FGF-6 was significantly decreased in non-metastatic cancer lesion tissues (**Figure 5C**) and the increased iron deposition (**Figure 5D, Figure S8B**). However, increased FGF-6expression was observed in metastatic liver carcinoma tissue (**Figure S9**), suggesting that FGF-6 plays different roles in oncogenesis and metastasis, analogous to TGF-.51,52

## Discussion

Iron homeostasis results from a combination of pathways and four main cell types: enterocyte, hepatocyte, macrophage, and erythroblast. The EGF/EGFR signaling pathway, heme production, STAT signaling, cAMP signaling, ferritin storage, and BMP-SMAD signaling are all involved in iron regulation. We conducted an exome-wide, gene-based recessive diplotype scan using putative functional variants to reveal additional genes underlying hemochromatosis susceptibility—an approach that can be widely applied to investigate complex disease susceptibility generated by compound heterozygosity and recessive single site effects using existing exome-wide association genotype and sequencing data. Although the case sample size was very small, this novel scan identified *FGF6* as being significantly associated with hemochromatosis following correction for multiple testing. *FGF6* belongs to the paracrine FGF-gene family and is largely expressed in skeletal muscle, which plays an important role in iron metabolism as it contains 10%-15% of iron stores. We conducted the evolutionary analysis of *FGF6* and known iron metabolism genes including *FGFR1, TFRC, FTH1, IREB1, TF, HMOX1, ACO2* and *HAMP* (encoding hepcidin). The appearance of iron metabolism genes can be separated into two stages. *TF* and *HMOX1*, which are found in animals from *C. elegans* to *H. sapiens*, indicate an origin in early Bilateria evolution (~635 Mya). *FGF6*, *FGFR1*, *ACO2* and *HAMP* can be found from *D. rerio* to *H. sapiens*, but are not present in *C. elegans* and *Drosophila*, indicating emergence in early Vertebrata (~485 Mya). The co-appearance of these genes suggests possible co-regulatory functions (**Figure S3A**). Functional experiments demonstrated that FGF-6 strongly impacted hepcidin expression to regulate iron homeostasis and decreases Fe2+ absorption in hepatocytes, while not impacting hepcidin-independent Fe3+ uptake. These results suggest FGF-6 mediates its effect on iron metabolism via hepcidin. The induction of hepcidin expression by FGF-6, presumably promotes ferroportin inhibition which was proposed as **Figure 6**. We additionally found that three *FGF6* nonsynonymous variants increased intracellular Fe2+ concentrations and reduced hepcidin levels compared to wildtype *FGF6*, indicating loss-of-function. Interestingly, a genome-wide RNAi profiling study reported that knockdown of *FGF6* increased transferrin-mediated endocytosis.53 Rs12368351, approximately 8kb downstream of *FGF6* has been associated with phosphorus levels;54 and two SNPs, rs140668749 and rs10849061, within 20kb downstream of *FGF6*, are associated with migraine.55,56 Previous studies have indicated that iron plays a role in autoimmunity and a study examining pulmonary arterial hypertension in SSc noted iron deposition in lung elastin fibers and giant cells57, however, epidermal iron deposition in SSc has not been previously investigated. We observed that FGF-6 is involved with iron deposition in SSc and liver cancer. Together, these results demonstrate that fibroblast growth factor receptor (FGFRs) signaling through FGF-6 is a critically important mechanism in iron metabolism.

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**Authorship Contributions**

SG performed analyses, interpreted results, designed the functional experiments, and aided in drafting the manuscript. SJ conducted molecular and cell biology experiments. MM aided in the analyses and reviewed the manuscript. MW, YM and WW provided clinical and biochemistry advice and aided in drafting the manuscript. MM aided with the experimental design and analyses. ZY performed initial genetic and statistical analyses, performed data management and reviewed the manuscript. BO implemented and refined the phenotyping algorithms. TK and JJ aided in the regulatory paperwork and reviewed the manuscript. RS performed data management tasks. JJM provided clinical advice and reviewed the manuscript. JKM supervised the management of biological samples for genotyping and reviewed the manuscript. LJ reviewed the manuscript and provided general scientific advice. JAS provided molecular and cellular biology advice, clinical advice, reviewed and edited the manuscript. JW supervised the functional experiments, reviewed the manuscript and provided biological advice. SJS designed the study, supervised the genetic analyses, developed phenotyping algorithms, developed analysis methods and power calculations, interpreted results and aided in drafting and editing the manuscript.

**Disclosure of Conflicts of Interest**

The authors declare no conflict of interest.

**Abbreviations**

GWAS: Genome-wide Association Studies

EHR: Electronic health record

PMRP: Personalized Medicine Research Project

PCA: Principal Components Analysis

AOD: Average optical density

HCT-8: Human ileocecal colorectal adenocarcinoma cell line

HCT116: Human colon carcinoma cell line

786-O: Human kidney adenocarcinoma cell line

HepG2: Human liver hepatocellular carcinoma cell line

HFF-1: Human skin fibroblast cell line

HBS: Heparin binding sites

HAMP: Hepcidin antimicrobial peptide

HEPH: Hephaestin

*FGF6*: Fibroblast Growth Factor 6 gene

FGF-6: Fibroblast Growth Factor 6 protein

M1: E172X *FGF6* variant (NM\_020996.2:c.514G>T)

M2: D174V *FGF6* variant (NM\_020996.2:c.521A>T)

M3: R188Q *FGF6* variant (NM\_020996.2:c.563G>A)

SSc: Systemic sclerosis

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## Tables

**Table 1:** **Significant genes identified by recessive diplotype scanning.**

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| CHR | GENE | P | OR# | SNPs | Case+ | Case- | Control+ | Control- |
| chr6 | *HFE* | 1.29×10−8 | 28.6 | 14 | 8 | 10 | 189 | 6707 |
| chr12 | *FGF6* | 1.99×10−6 | 22.8 | 10 | 6 | 12 | 153 | 6743 |
| chr21 | *KRTAP15-1* | 7.55×10−5 | 6.78 | 5 | 11 | 7 | 1271 | 5625 |
| chr20 | *XKR7* | 1.18×10−4 | 43.6 | 7 | 3 | 15 | 35 | 6861 |
| chr20 | *CABLES2* | 1.28×10−4 | 42.4 | 7 | 3 | 15 | 36 | 6860 |
| chr22 | *THOC5* | 1.38×10−4 | 6.24 | 9 | 13 | 5 | 1945 | 4951 |

The six most significant genes identified in the recessive diplotype scan are displayed. P-values are from a two-tailed Fisher’s exact test. OR# shown as Haldane odds ratio. SNPs: number of genotyped variants per gene that were polymorphic in the samples studied. Case+: number of iron overload case individuals carrying recessive diplotypes with putative functional alleles. Case-: number of cases carrying at least one homolog at the gene without a putative functional allele. Control+: number of control individuals carrying recessive diplotypes with putative functional alleles. Control-: number of controls carrying at least one homolog at the gene without a putative functional allele.

## Figure legends

**Figure 1.** **Manhattan plot of the gene-based recessive diplotype association results.** The association *P*-value testing hemochromatosis association for each gene (y-axis) on different chromosomes is shown in alternating navy blue and green along the x-axis, with genes reaching experiment-wise significance for the gene-based analyses (experiment-wise  = 3.14 × 10−6) depicted in red.

**Figure 2.** **Comparative genomic analysis.** The comparative genomic analyses revealed that *FGF6* evolved synchronously with other iron metabolism genes. **(A)** Main iron metabolism genes were collected and alignment was conducted to make the comparative genomic analysis together with *FGF6*. The earliest gene appearance over time was inferred by comparing species and corresponding evolution and appearance time was labelled. **(B)** Protein-protein interaction network was estimated by String (version 10.0)58 using the highest confidence setting (confidence score>0.9). **(C)** Protein domain summarized by a previous FGF-6 functional study59. HBS: Heparin Binding Sites; FGFR-BR: FGFR-Binding Region.

**Figure 3.** **FGF-6 regulates hepcidin-dependent iron uptake.** Prussian blue staining was applied for the evaluation of iron deposition. Fe2+, Fe3+ as well as FGF-6 active proteins were added into the cell culture medians, respectively, with different recombination. Control group was treated with vehicle alone (boiled water). Iron staining was visualized by microscopy. Iron positive staining cells divided by total cell number was used to evaluate the iron deposition levels.

**Figure 4.** ***FGF6* loss-of-function nonsynonymous variants cause hepcidin downregulation and iron deposition. (A)** Prussian blue staining to HepG2 cells with different nonsynonymous mutations using Fe2+ and Fe3+ treatment. **(B)** quantitation of Iron absorption difference between *FGF6* plasmid transfection with wildtype, M1 (E172X), M2 (D174V), M3 (R188Q) mutation. Iron staining was visualized by microscopy. Iron positive staining cells divided by total cell number was used to evaluate the iron deposition levels. **(C)** Iron metabolism gene expression changes with FGF-6 active protein treatment in the cell culture media and *FGF6* mRNA transfection. **(D-G)** Iron metabolism gene expression changes after the transfection by *FGF6* mRNA into various cell types with wildtype and the identified variants R188Q, D174V and E172X. Cell lines: 786-O are kidney adenocarcinoma cells, A-498 are kidney carcinoma cells, HCT-8 are ileocecal colorectal adenocarcinoma cells, and HepG2 are liver hepatocellular carcinoma cells.

**Figure 5.** **Decreased *FGF6* expression gene in systemic sclerosis and hepatic cancer.** **(A)** FGF-6 protein level was evaluated by immunohistochemistry assay (IHC) in skin tissues from SSc patients. **(B)** Prussian blue staining was applied to evaluate the iron deposition in SSc skin tissues. **(C)** Immunohistochemistry assay of FGF-6 protein in liver cancer tissues. **(D)** Prussian blue staining was applied to evaluate the iron deposition in liver cancer tissues. FGF-6 expression level (Immunohistochemistry,IHC,staining) were counted by average optical density (AOD) of positive signal in each sample. Iron staining was visualized by microscopy. The ratio of iron-positive stain areas to the total area was used to evaluate the iron deposition levels by Image J software.

## Analysis Code:

We uploaded the Code to github: <https://github.com/Shicheng-Guo/marshfield/blob/master/2ALOF/readme.md>