Identification of Novel Non-HFE Mutations in Chinese Patients with Hereditary Hemochromatosis

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Abstract

Backgrounds

Hereditary hemochromatosis (HH) is mainly caused by homozygous p.C282Y mutations in HFE in the Caucasians. We recently reported non-HFE mutations constitute the major cause of HH in Chinese. However, there is still a relatively high proportion of cases with primary iron overload from unexplained causes. We aimed to explore novel non-HFE mutations in Chinese patients with primary iron overload.

Methods

Whole exome sequence was conducted to screen mutations in novel HH-related genes in the 9 cases with unexplained primary iron overload. Then the representative candidate genes were screened for mutations in another cohort of 18 HH cases. The biological function of the selected genes and variants were analyzed in vitro.

Results


Conclusions

Our study identified a series of novel candidate non-HFE mutations in Chinese patients with HH. These may provide insights into the genetic basis of unexplained primary iron overload.

Introduction

Hereditary hemochromatosis (HH) is characterized by excessive iron deposition in the liver and other organs including heart, pancreas, bone and joints, and pituitary gland, leading to hepatic and extra-hepatic complications. This disorder is related to deficiency or resistance of hepcidin, which is produced by hepatocytes in the liver and blocks the entry of iron into the plasma by inducing the internalization and degradation of the iron exporter ferroportin (FPN1) [1]. The known causes of HH include gene mutations in upstream regulators of the expression of hepcidin, such as human hemochromatosis protein (HFE), hemouvelin (HJV), hepcidin gene (HAMP), transferrin receptor-2 (TFR2), and its target FPN1 (also known as SLC40A1)[2].

In Caucasians patients with HH HFE p.C282Y is the most common mutation, accounting for more than 90% of cases. Non-HFE-related HH accounts for a minority of the remaining cases. However, some patients with primary iron overload do not show mutations in the aforementioned genes. Studies with next generation whole exome sequencing (NGWES) found that mutations in the pro-peptide domain region of BMP6 and mutations in NMBR were new genetic factors predisposing to primary iron overload [3, 4].


However, there are still some cases with primary iron overload had undefined genetic causes (approximately 30% of patients in the China Registry of Genetic/Metabolic Liver Diseases; data not shown). Therefore, in the present study, we explored novel HH-related genes by whole exome sequencing (WES) in a cohort of cases with primary iron overload with unexplained causes. We first identified mutations in a series of potential iron metabolism–related genes such as ubiquitin-conjugating enzyme E2 0 (UBE2O), proprotein convertase subtilisin/kexin type 7 (PCSK7), then we analyzed the function and explored the mechanisms of representative genes and variants.

Patients And Methods

Patients

Patients with primary iron overload were enrolled at the China Registry of Genetic/Metabolic Liver Diseases for the genetic analysis of mutations in HH-related genes.

The diagnosis of HH was based on the American Association for the Study of Liver Diseases 2011 practice guidelines on hemochromatosis[13] as follows: 1) transferrin saturation (TS) ≥ 45% and/or elevated ferritin (>300 ng/mL in men and postmenopausal women or >200 ng/mL in premenopausal women); 2)
iron overload in the liver and/or spleen on magnetic resonance imaging of the liver or liver histology; and 3) excluded causes of secondary iron overload, such as alcoholic or other chronic liver disease, iron-overloading anemia, and parenteral iron overload.

This study was approved by the Clinical Research Ethics Committee of Beijing Friendship Hospital, Capital Medical University (No. 2016-P2-061-01). Informed and written consent was obtained from all patients.

Blood sample collection and DNA extraction

Genomic DNA was extracted from whole blood using a Genomic DNA Purification Kit (Qiagen, Valencia, CA, USA). Quality control was performed by evaluating the 260/280 nM absorbance ratio and gel electrophoresis.

Identification of novel mutations in the discovery cohort by WES

WES was performed on DNA extracted from the peripheral blood of nine unrelated patients with primary iron overload, in which mutations in known HH-related genes (HFE, HJV, HAMP, TFR2, SLC40A1) could not explain the severity of iron overload. Table 1 summarizes the clinical features of these patients.

A targeted exome library with an insert size of 150–200 bp was constructed from approximately 1 μg of genomic DNA by an exome capture strategy using a GenCap custom exome enrichment kit (MyGenostics, Beijing, China). The Illumina HiSeq 2000 platform was used to generate paired-end 100 bp raw reads from each enriched library according to the manufacturer's protocol. The 100 bp paired-end reads were aligned against NCBI build 37 of the human genome using Burrows Wheeler Aligner. Duplicate reads were marked, local indel realignment performed and base quality scores were recalibrated for each sample with the Genome Analysis Toolkit.

Novel point mutations were identified using MuTect, while indel variants were identified using Somatic Indel Detector in the Genome Analysis Toolkit. The potential pathogenic variants were confirmed by Sanger sequencing.

The criteria for the screening of the mutations in potential iron metabolism–related genes were as follow: in public databases, the population frequency is less than 1%, no report or prediction as benign or likely benign, and predicted as disease causing by at least one of the prediction tools SIFT, Polyphen-2 and Mutation Taster.

Screening for the newly discovered gene mutations in a cohort of primary iron overload by Sanger sequencing

To investigate the novel candidate genes, we enrolled 18 unrelated patients with HH as the validation cohort. Among the 18 patients, 3 carried HFE mutations, 7 carried HJV mutations, 3 carried TFR2 mutations, 7 carried SLC40A1 mutations, 2 carried SUGP2 mutations, 1 patient carried TMPRSS6 mutation, and 1 patient carried BMP4 mutation. The clinical features of these 18 cases are shown in Table S1.

All exons of UBE2O and PCSK7 were PCR-amplified with associated boundary regions using specific primers (see Table S2). PCR amplification was performed in an ABI Veriti 96 PCR cycler (Applied Biosystems, Foster City, CA, USA). PCR products were sequenced using an automated ABI 3730 DNA sequencer (Applied Biosystems).

Functional analysis of the newly discovered gene mutations

Cell culture and transfection

The human hepatocellular carcinoma (HCC) cell lines Huh-7 and HepG2 were obtained from the Cell Resource Center of the Chinese Academy of Medical Science (Beijing, China). Huh-7 and HepG2 cells were cultured as described previously[5,14].

For the siRNA-mediated transient knockdown of gene expression, Huh-7 and HepG2 cells (5×10^5 cells) were transfected with 20 nM siRNA using LTX reagent (Invitrogen, USA) in accordance with the manufacturer's instructions.

For the adenovirus generation and establishment of stable UBE2O-overexpressing and UBE2O-K689R cell lines, Huh-7 and HepG2 cells were infected with the adenovirus. After 24h, we confirmed infection by observed expression of red fluorescence protein. After 24h, assays were performed.

siRNA interference of UBE2O and PCSK7

Gene knockdown was performed using UBE2O siRNA (ID siG000063893), PCSK7 siRNA (ID siG000009159), and the negative control siRNA (ID siN0000001-1-5) (RiboBio, Guangzhou, China).

Adenovirus generation for UBE2O and UBE2O-K689R expression

The UBE2O and UBE2O p.K689R sequences were cloned into the pADV-EF1-mScarlet-CMV-MCS-3xFLAG vector, and Huh-7 and HepG2 cells were infected with the adenovirus.

Real-time PCR

The isolation of total RNA from cell lines and real-time PCR assays were conducted as described previously[11]. The primer sequences are listed in Supplementary material-Table S2. GAPDH mRNA served as control.

Immunofluorescence staining
Immunofluorescence analysis was conducted as described previously[5,14]. Cells were incubated with a primary antibody directed against rabbit anti-hepcidin (1:50; Abcam, USA) at 4°C overnight. After three 5 min washes with phosphate-buffered saline (PBS), cells were incubated with anti-rabbit Alexa 488-conjugated secondary antibodies (1:500; Invitrogen) for 1 h at room temperature. After additional PBS washes, cells were mounted on a slide in mounting medium (Molecular Probes). Cells were then examined and photographed using an FV 300 confocal microscope (Olympus, Tokyo, Japan).

Western blotting

Western blot analysis was performed as described previously[5,14]. Membranes were incubated with rabbit anti-UBE2O (1:1000; Novus), rabbit anti-Smad6 (1:1000; BD), rabbit anti-p-Smad1/5 (1:1000; Sigma), mouse anti-Smad7 (1:100; RD) or mouse anti-GAPDH antibodies (1:5000; Zhongshan jinqiao) overnight at 4°C, followed by incubation with horseradish peroxidase–conjugated goat anti-mouse or anti-rabbit antibodies (1:5000; Zhongshan jinqiao) for 1 h at room temperature. Target proteins were detected using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA).

Enzyme-linked immunosorbent assay (ELISA)

The human hepcidin detection kit (Cloud-Clone Corp, SEB979Hu) was used to determine the concentration of hepcidin in cell culture supernatants according to the manufacturer's protocol.

Statistical analysis

All experiments were carried out at least three times. The Fisher's exact test was used to determine the difference of mutation frequency between patients and healthy subjects using SPSS v21.0 software. P < 0.05 was considered to indicate statistical significance.

Results

Identification of novel non-HFE variants in primary iron-overload cases by WES

We performed WES in 9 unrelated patients with unexplained primary iron overload. Based on the criteria as described in the methods, we identified a total of 69 mutations in 61 genes associated with iron metabolism pathway (Supplementary material 1), including 42 missense variants in 40 genes, such as UBE2O, PCSK7, COL6A5, MRRF and MUC5B genes (Supplementary material 2).

Screening of UBE2O and PCSK7 variants in patients with primary iron overload cohort by Sanger sequencing

We next performed Sanger sequencing for the two identified genes in 27 primary iron-overload cases. Two cases carried UBE2O p.K689R (7.4%, 2/27); one case carried p. R711W (3.7%, 1/27) and two cases carried p. V143F (7.4%, 2/27) in PCSK7 (Fig.1A–B, Table 2, Table S3-4). In addition, 116 unknown potential iron metabolism–related genes were identified with mutations in at least two of the nine cases, such as COL6A5, MRRF and MUC5B genes (Supplementary material 2).

HAMP expression was decreased in UBE2O- and PCSK7-knockdown HCC cells

We next conducted in vitro siRNA interference of representative genes UBE2O, PCSK7 to analyze their effects on HAMP mRNA expression. Results from qRT-PCR showed that the level of HAMP mRNA, which encodes hepcidin, was decreased in UBE2O- and PCSK7-knockdown HCC cells compared with control cells (Fig.2A, B). These results indicate that knockdown of UBE2O, or PCSK7 gene reduces the expression of HAMP mRNA.

The inhibition of hepcidin expression by UBE2O p.K689R was through inhibiting Smad1/5 phosphorylation by enhancing the function of Smad6 and Smad7

It has been known UBE2O monoubiquitinates inhibitory-Smads (Smad6 and Smad7), blocking its association with activated type I receptor BMPRI and resulting in increased BMP/SMAD signaling[15] We used UBE2O p.K689R as a representative for initial mechanism study. Results from qRT-PCR showed that mutant UBE2O (p.K689R) down-regulated HAMP mRNA level to a greater extent than wild-type UBE2O in HepG2 cells and Huh-7 cells (Fig.3A). Consistent with the reduced expression of HAMP mRNA caused by UBE2O p.K689R, the hepcidin level in cells expressing mutant UBE2O was lower than cells expressing wild-type UBE2O in ELISA and immunofluorescence staining assays (Fig.3B and C).

Western blot analysis revealed higher expression of Smad6 and Smad7 and lower expression of pSmad1/5 in UBE2O p.K689R HCC cells than wild-type HCC cells (Fig.3D). These results indicate that the inhibition of hepcidin expression by UBE2O p.K689R may be through inhibiting ubiquitination-mediated degradation of Smad6 and Smad7, and the subsequent inhibition of SMAD1 phosphorylation.

Discussion

In the present study, we firstly identified novel mutations by NGWES in a small number of patients with unexplained primary iron overload, then we screened for the mutations in the representative newly identified genes, UBE2O and PCSK7, in a larger cohort of primary iron overload patients. We found recurrent p.K689R variant in UBE2O and a high frequency of mutations in PCSK7 in primary iron overload patients. Functional studies indicated that mutation in UBE2O and PCSK7 may play a role in the regulation of HAMP/hepcidin expression. Taken together, our study identified a series of novel candidate non-HFE mutations in Chinese patients with HH, and as the representative candidate, the UBE2O and PCSK7 may function in iron metabolism, which is essential for the HH cases that are difficult to interpret.

UBE2O (Uniprot accession #Q9C0C9) is encoded at the 17q25 region. As an E2/E3 hybrid ubiquitin-protein ligase[16], UBE2O displays roles of an E2 ubiquitin conjugating enzyme and E3 ubiquitin ligase. UBE2O contains three conserved regions (CR1, CR2, and CR3), a coiled-coil domain, a UBC domain, and two
putative nuclear localization sequences [17]. UBE2O acts on a broad spectrum of targets and execute multiple biological functions. For example, UBE2O negatively regulates TRAF6-mediated NF-kB activation by inhibiting TRAF6 polyubiquitination[18]. UBE2O multi-monoubiquitates the nuclear localization signal of BAP1, inducing its cytoplasmic sequestration[17]. UBE2O promotes the proliferation, epithelial-mesenchymal transformation, and stemness properties of breast cancer cells through the UBE2O/AMPKα2/mTORC1 positive feedback loop[19], and UBE2O facilitates tumorigenesis and radiosensitivity by promoting Mxi1 ubiquitination and degradation[20]. Thus, UBE2O targets several proteins for ubiquitination and has been implicated in chromatin-associated protein nuclear transport, adipogenesis, tumor progression, and metastasis[16, 18–22]. However, no study has linked UBE2O to the regulation of iron metabolism. In the present study, we found that two HH patients carried p.K689R in UBE2O. However, the mechanism of UBE2O regulating iron metabolism is not clear.

Hepcidin deficiency is the common feature in HH and is responsible for iron overload in HH. Hepcidin acts in close connection with ferroportin to regulate iron metabolism. When plasma or hepatocyte iron concentrations increase, signaling pathways including ERK/MAPK and BMP/SMAD pathways are activated and induce hepcidin mRNA expression, leading to increased plasma hepcidin. Hepcidin then interacts with ferroportin, resulting in decreased duodenal iron absorption and a decreased release of iron from the spleen coming from erythrophagocytosis [1]. The BMP/SMAD pathway is the major pathway for transcriptional regulation of hepcidin expression in hepatocytes. SMAD6 and SMAD7 are inhibitory SMADs that are induced by BMP/SMAD signaling and inhibit the BMP/SMAD pathway by interfering with type I receptor function or SMAD complex formation[2]. Zhang and colleagues found that UBE2O monoubiquitimates SMAD6, blocking its association with activated type I receptor and resulting in increased BMP/SMAD signaling[15]. Furthermore, the authors showed that forced UBE2O expression in C3H10T1/2 cells potentiated BMP7-induced SMAD1 phosphorylation and adipocyte differentiation, and forced UBE2O expression in C2C12 cells enhanced BMP6-induced SMAD1 phosphorylation and osteoblast differentiation [15]. This indicates that UBE2O may regulate the BMP/SMAD pathway through the ubiquitination of I-SMADs.

In our study, we found increased expression of Smad6 and Smad7 and decreased expression of HAMP mRNA in UBE2O-knockdown cells, while the expression of p-SMAD1/5 increased in UBE2O-knockdown cells. In UBE2O p.K689R-expressing cells, the expression of SMAD6 and SMAD7 increased and the expression of p-SMAD1/5 decreased, along with reduced expression of HAMP mRNA. This suggests that UBE2O may influence iron metabolism by regulating the BMP/SMAD pathway. We speculate that UBE2O regulates the BMP/SMAD pathway through ubiquitination of SMAD6 and SMAD7, and we will explore this hypothesis in future research.

We also identified high frequency of PCSK7 variations in patients with primary iron overload. PCSK7 is a family member of nine secretory serine proteases related to bacterial subtilisin and yeast kexin (PCSK1–PCSK9)[23]. Oexle et al. established a strong link between plasma levels of the soluble human transferrin receptor 1 and PCSK7 by a genome-wide association study[24]. Guillenot et al. later found that PCSK7 acted in iron homeostasis by directly shedding HTR1 by cleavage at an atypical site and showed that furin alone activates hepcidin[25]. Our study revealed that HAMP mRNA was decreased in PCSK7-knockdown HCC cells, further indicating that PCSK7 may be a candidate gene or modifier gene causing iron overload involved in HH.

This study has several limitations. First, this study lacked in vivo data, and animal models are needed to clarify the effect of UBE2O on iron metabolism. Second, the mechanisms by which PCSK7 impact hepcidin expression are unknown, and thus the molecular signaling involving hepcidin regulation by PCSK7 needs further study. Finally, the genetic association needs further validation in a larger cohort of patients with primary iron overload.

In conclusion, our study identified a series of novel candidate non-HFE mutations in Chinese patients with HH, which may provide insights into the genetic basis of the unexplained primary iron overload.

**Abbreviations**

HH, hereditary hemochromatosis; HFE, human hemochromatosis protein; HJV, hemojuvelin; HAMP, hepcidin antimicrobial peptide; SLC40A1, solute carrier family 40 member 1; FPN1, ferroportin 1; BMP, bone morphogenetic protein; SMAD, small mothers against decapentaplegic; UBE2O, ubiquitin-conjugating enzyme E2 O; PCSK7, proprotein convertase subtilisin/kexin type 7; TS, transferrin saturation; PBS, phosphate-buffered saline; NGWES, next generation whole exome sequencing.

**Declarations**

**Acknowledgements**

We are grateful to the patients and their families for their supports and participation in this study.

**Author’s Contributions**

WZ, YL conceived the study, performed the experiments, carried out the data analysis, imaging, image analysis, bioinformatics analysis and draft the manuscript. AX, QO, LW, DZ, LW and BZ performed the experiments, carried out the data analysis and bioinformatics analysis. XZ, YW, XW, WD, QW and HY contributed to the acquisition, analysis and interpretation of the data. JH, XO and JJ made substantial contributions to conception and design and involved in revising the manuscript critically for important intellectual content. All the authors approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical approval and consent to participate

This study was approved by the Clinical Research Ethics Committee of Beijing Friendship Hospital, Capital Medical University (No. 2016-P2-061-01). Informed and written consent was obtained from all patients. The study was registered with the ClinicalTrials.gov identifier NCT03131427.

Consent for publication

All authors agreed on the manuscript.

Competing interests

All authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Details

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On behalf of China Registry of Genetic/Metabolic Liver Diseases (CR-GMLD) Group

References


### Tables

**Table 1. Clinical Characteristics of discovery cohort with primary iron overload**

<table>
<thead>
<tr>
<th>Age</th>
<th>Sex</th>
<th>SF (ng/ml)</th>
<th>TS (%)</th>
<th>AST (U/L)</th>
<th>ALT (U/L)</th>
<th>T-Bil (umol/L)</th>
<th>D-Bil (umol/L)</th>
<th>γ-GGT (U/L)</th>
<th>Iron overload on MRI</th>
<th>Iron overload on liver biopsy</th>
<th>End-organ manifestations</th>
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<tr>
<td>D1</td>
<td>62</td>
<td>F</td>
<td>5346</td>
<td>99</td>
<td>49</td>
<td>54</td>
<td>9</td>
<td>2</td>
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<tr>
<td>D2</td>
<td>28</td>
<td>M</td>
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<td>192</td>
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<td>Skin pigmentation, liver cirrhosis, diabetes, amenorrhea</td>
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<td>M</td>
<td>2000</td>
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<td>109</td>
<td>215</td>
<td>32</td>
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<td>291</td>
<td>Liver, spleen</td>
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<td>M</td>
<td>685</td>
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<td>8</td>
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<td>Liver</td>
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ALT, alanine aminotransferase; AST, aspartate aminotransferase; ND: Not done; SF, serum ferritin; T-Bil, total bilirubin; D-Bil, direct bilirubin; TS, transferrin saturation; γ-GGT, gamma glutamyl transpeptidase; MRI, magnetic resonance imaging

**Table 2. UBE2O and PCSK7 missense mutations identified in patients with primary iron overload**

<table>
<thead>
<tr>
<th>Gene (accession number)</th>
<th>Amino acid change</th>
<th>Base change</th>
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<th>gnomAD_exome</th>
<th>Polyphen-2 Prediction</th>
<th>SIFT Prediction</th>
<th>Mutation Taster Prediction</th>
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<td>UBE2O (NM_022066)</td>
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<td>c.2066A&gt;G</td>
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<td>Tolerable 0.853</td>
<td>Disease causing 1.0</td>
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<td>PCSK7 (NM_004716)</td>
<td>p.R711W</td>
<td>c.2131C&gt;T</td>
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Figures

Figure 1

Representative sequencing of the novel variants in *UBE2O* and *PCSK7* in cases with primary iron overload. Sequencing of the heterozygous missense mutations *UBE2O* p.K689R (A) and *PCSK7* p.R711W (B).

Figure 2

Analysis of *HAMP* mRNA expression in *UBE2O* and *PCSK7* knockdown cells. A. *HAMP* mRNA levels in Huh-7 and HepG2 cells transfected with *UBE2O* siRNA or control siRNA. B. *HAMP* mRNA levels in Huh-7 and HepG2 cells transfected with *PCSK7* siRNA or control siRNA.

Figure 3

Analysis of *HAMP, SMAD6*, and *SMAD7* expression in Huh7 and HepG2 cells infected with *UBE2O* or *UBE2O* p.K689R adenovirus. A. *HAMP* mRNA levels in Huh-7 and HepG2 cells were analyzed by real-time PCR assays. B and C. Hepcidin was analyzed in Huh-7 and HepG2 cells by immunofluorescence and ELISA. D. Western blot analysis of SMAD6, SMAD7, and pSmad1/5 expression in Huh-7 and HepG2 cells.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarymaterial1.xlsx
- Supplementarymaterial2.xlsx
• TableS1ClinicalCharacteristicsofvalidationcohort.docx
• tableS2PrimersfortheSangersequencing.docx
• TableS3Qualitycontrolofthewholeexomesequen.docx
• TableS4.MissensevariantsidentifiedbytheNGWS.docx