

Type 4B hereditary hemochromatosis associated with a novel mutation in the *SLC40A1* gene

A case report and a review of the literature

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Abstract

Rationale: Hereditary hemochromatosis can be divided into *HFE*- and non-*HFE*-related based on genetic mutations in different genes. *HFE*-related hemochromatosis is the most common inherited genetic disease in European populations but rare in Asia-Pacific region. Recently, non-*HFE*-related hemochromatosis has been reported in patients from the Asian countries.

Patient concerns: We report the case of a 48-year-old Chinese Han woman who presented with abnormal liver function, diabetes mellitus, hyperferritinemia, and high transferrin saturation, with severe iron overload in parenchymal cells, Kupffer cells, and periportal fibrosis on liver biopsy. No secondary factor for iron overload was identified.

Diagnoses: Sanger sequencing was conducted for the screening of mutation in the hereditary hemochromatosis related genes. The functional effect of a splicing mutation, *SLC40A1* IVS 3+10 del gtt, was assessed by reverse-polymerase chain reaction analysis for *SLC40A1* mRNA level, and by immunohistochemistry analysis of liver biopsy for ferroportin expression and cellular localization.

Outcomes: A novel splicing mutation IVS 3+10 del gtt was identified in the *SLC40A1* gene. Functional analysis showed that IVS 3+10 del gtt in the *SLC40A1* gene lead to a substantial reduction in the basal levels of *SLC40A1* mRNA and increased membrane localization of ferroportin. Finally, the patient was diagnosed as ferroportin disease (type 4B hemochromatosis).

Lessons: The present study is the first report to identify a classical splicing mutation in the *SLC40A1* gene in type 4B hemochromatosis, and provide further evidence of the prevalence of type 4 hereditary hemochromatosis in Asian countries.

Abbreviations: BSA = albumin from bovine serum, GRE = gradient-echo, *HAMP* = hepcidin antimicrobial peptide, *HFE* = hemochromatosis, HH = hereditary hemochromatosis, *HJV* = hemojuvelin, IHC = immunohistochemistry, MRI = magnetic resonance imaging, PBS = phosphate buffer saline, RT-PCR = reverse transcriptase-polymerase chain reaction, *SLC40A1* = solute carrier family 40 member 1, *TFR2* = transferrin receptor 2.

Keywords: case report, hemochromatosis, iron overload, liver, *SLC40A1*, splicing mutation

1. Introduction

Hereditary hemochromatosis (HH) is a genetic disorder characterized by iron deposition and tissue injury in multiple organs.^[1] The genetic bases for hemochromatosis can be divided principally into hemochromatosis (*HFE*) gene mutations and non-*HFE* mutations.^[2] The principal *HFE* gene defect was first

identified in 1996, and is a G-to-A missense mutation, leading to the substitution of tyrosine for cysteine at amino acid position 282 of the protein product (C282Y); the other 2 relatively common *HFE* mutations were H63D and S65C.^[3] *HFE*-related HH is the most common inherited genetic disease in European populations.^[1] Mutations in all the currently known genes implicated in non-*HFE* HH, such as hemojuvelin (*HJV*), hepcidin antimicrobial peptide (*HAMP*), transferrin receptor 2 (*TFR2*), and solute carrier family 40 member 1 (*SLC40A1*), have been reported in patients from the Asia-Pacific region.^[2] Further, a meta-analysis found that 80.6% (2260/2802) of HH patients are homozygous for the C282Y mutation of *HFE*,^[3] yet this mutation is not found in Chinese patients.^[4] Here, we report a novel *SLC40A1* mutation, IVS 3+10 del gtt, in a Chinese Han woman with severe iron overload disease.

2. Clinical report

2.1. Patient identification

A 48-year-old female patient who had been suffering from asthenia and intermittent vomiting for 9 months was admitted to our hospital. She had no other gastrointestinal symptoms, but had been diagnosed with diabetes mellitus for 2 years. She had never taken supplemental iron and did not consume alcohol. Physical examination was normal. Laboratory biochemistry results were as follows: alanine aminotransferase (ALT) 62 U/L (normal: 7–40 U/L); aspartate aminotransferase (AST) 68 U/L (normal: 13–35 U/L); alkaline phosphatase (ALP), 113 U/L

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The authors declare no conflict of interest.

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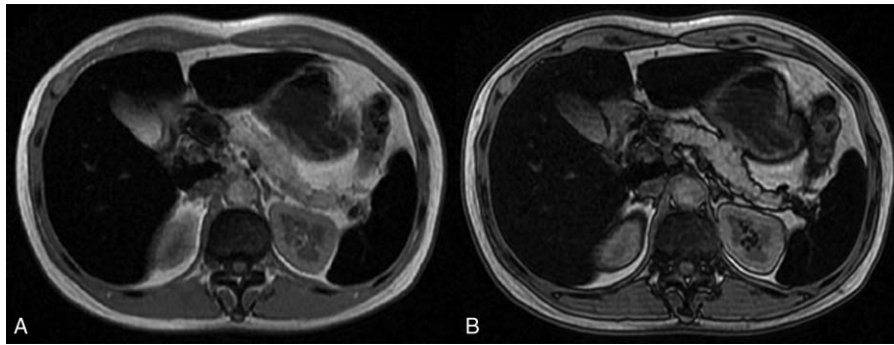


Figure 1. Dual-sequence magnetic resonance (MR) imaging of the liver. Axial GRE T1-weighted in-phase (A) and out-of-phase (B) MR images show a significant decrease in signal intensity of the liver and spleen on the in-phase image ($T2^*$ effect). GRE = gradient-echo.

(normal: 35–100 U/L); γ -glutamyltransferase (GGT), 49 U/L (normal: 7–45 U/L); and total and conjugated bilirubin (TB/DB), 7.35 and 0.86 $\mu\text{mol/L}$, respectively. Serum markers for viral hepatitis were negative. Antimitochondrial antibody (M2) and immunoglobulin M were normal. Ceruloplasmin was 0.20 g/L (normal: 0.20–0.60 g/L). Iron studies showed: iron 34.34 $\mu\text{mol/L}$, transferrin saturation 100%, and ferritin 7078 $\mu\text{g/L}$. Magnetic resonance imaging (MRI) of the abdomen showed a significant iron overload in the liver and spleen (Fig. 1). A liver biopsy was performed for diagnostic purposes and staging of her liver disease. The specimen obtained was prepared with hematoxylin/

eosin and Berlin blue staining. Histological examination of the specimen showed grade 3 iron stores (using the Scheuer scoring system), predominantly in parenchymal cells, but with significant iron deposits in Kupffer cells and portal tract macrophages (Fig. 2). In addition, periportal fibrosis was observed, but no cirrhosis was evident.

2.2. Detection of genetic mutations

Informed consent was obtained before taking a blood sample for gene analysis of *HFE* (C282Y and H63D), *HJV*, *HAMP*, *TFR2*,

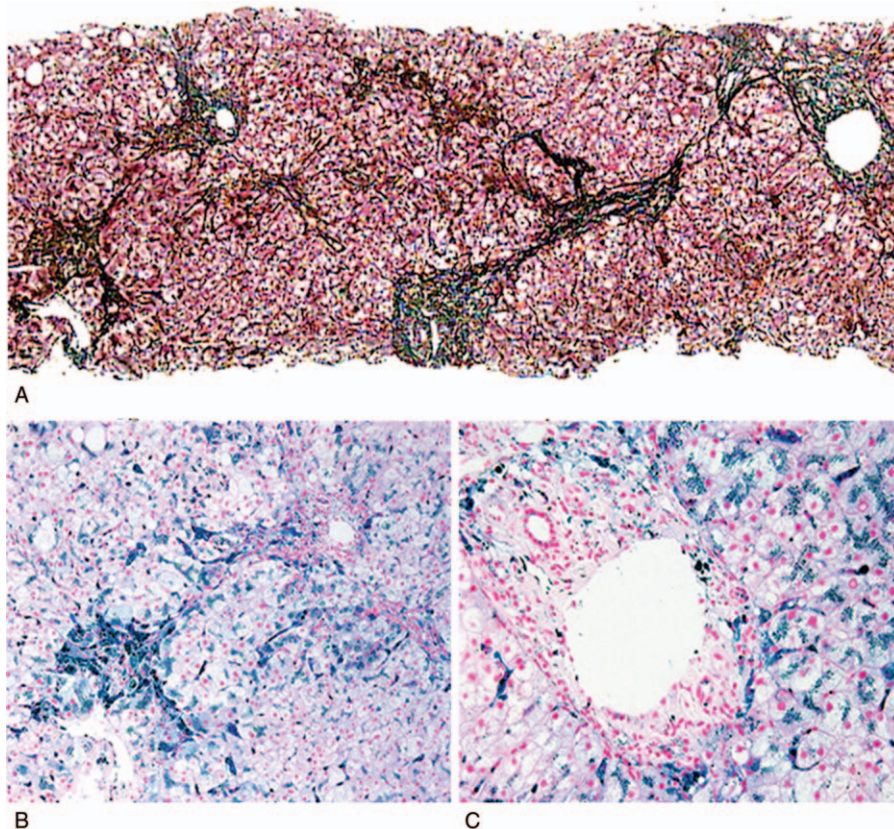


Figure 2. Liver biopsy sections showing periportal fibrosis and iron overload. Liver biopsy showing (A) periportal fibrosis and (B, C) heavy (grade 3) parenchymal iron accumulation with significant Kupffer cell and macrophage siderosis. (A) reticulin + Masson, 4 \times magnification; (B) Perls, 20 \times magnification; (C) Perls, 40 \times magnification.

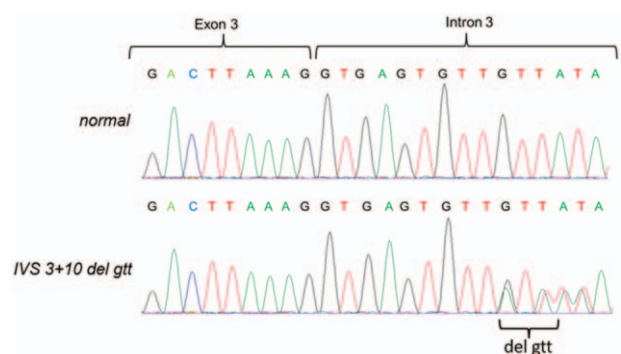


Figure 3. Sequence analysis of the *SLC40A1* gene showing the heterozygous splice site mutation, IVS3 +10 del gtt.

and *SLC40A1*. Efforts were made to contact and obtain blood samples from family members. However, owing to circumstances beyond our control, none could be contacted.

Genomic DNA was extracted from peripheral blood leukocytes using the Genomic DNA Purification Kit (Qiagen, Valencia, CA). Whole exons were amplified from the *HFE*, *HJV*, *HAMP*, *TFR2*, and *SLC40A1* genes, and their associated boundary regions by polymerase chain reaction (PCR) using the primers described in our previous study.^[5] Amplification was performed with an initial denaturation of 1 cycle of 95°C for 5 minutes, followed by 38 cycles of 95°C for 1 minute, annealing at 50 to 60°C for 70 seconds, and 72°C for 5 minutes. PCR products were sequenced using the ABI3730 automated DNA sequencer. The cDNA reference sequence was submitted to GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>) and analyzed by ExpASY

(<http://web.expasy.org/translate/>). The results showed that the patient had a splicing mutation, IVS3+10 del gtt, in the *SLC40A1* gene (Fig. 3). No mutations were identified in the other 4 genes.

2.3. Reverse transcriptase-PCR (RT-PCR) analysis of *SLC40A1* gene transcripts

Total RNA was isolated from formalin-fixed paraffin-embedded tissue of the patient using the RNeasy FFPE Kit (Qiagen, Valencia, CA) according to the manufactory's protocol, and the reverse-transcript was conducted using the High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster city, CA). cDNA of BEL7402 cell line and normal liver tissue were provided by Dr Anjian Xu (Experimental Center, Beijing Friendship Hospital, Capital Medical University, Beijing, China) and used as control for RT-PCR analysis. RT-PCR covering the coding sequence of exons 3 to 4 of *SLC40A1* was performed with the primers as follows: 5'-TTGCGGTGTCTGTGTTCTG-3' (in exon 3) and 5'-AGGTCTGGGCCACTTTAAGT-3' (in exon 4) for *SLC40A1*, and 5'-GAGTCAACGGATTTGGTCGT-3' and 5'-GAGTCAACGGATTTGGTCGT-3' for *GAPDH* as control. The reactions were performed in an ABI GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA) with the following program: denaturation for 30 seconds at 94°C, annealing for 30 seconds at 60°C, and elongation for 30 seconds at 72°C for 35 cycles; the final extension at 72°C for 7 minutes. Equal volumes of each PCR sample were subjected to electrophoresis in a 1.5% agarose gel, and the PCR product of *SLC40A1* was conducted sequencing with the same primer for PCR.

The results showed a substantial reduction in the basal levels of *SLC40A1* mRNA in the case with *SLC40A1* IVS3+10 del gtt mutation compared with that of the controls (Fig. 4A), suggesting

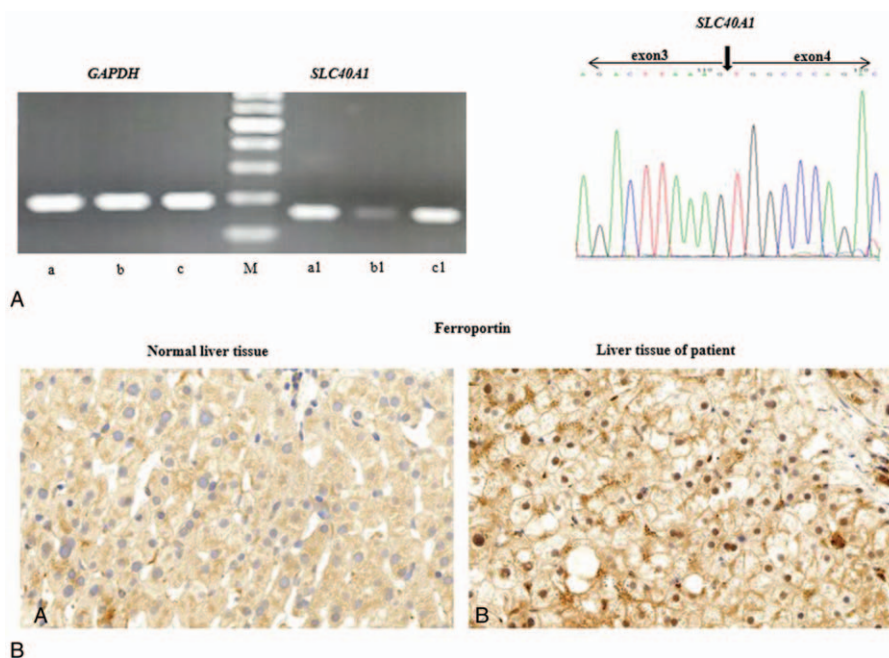


Figure 4. RT-PCR analysis of *SLC40A1* gene transcripts and immunohistochemical analysis of ferroportin in liver tissue of the HH patient with *SLC40A1* IVS3+10 del gtt mutation. (A) RT-PCR analysis of *SLC40A1* gene transcripts of the patient, with transcripts of BEL7402 cell line and normal liver tissue as control. a/a1 = BEL7402 cell line, b/b1 = patient with *SLC40A1* IVS3+10 del gtt mutation, c/c1 = normal liver tissue, for detection of *SLC40A1* with *GAPDH* as loading control, M = 100 bp DNA ladder. (B) Immunohistochemical analysis of ferroportin in liver tissue of the HH patient with *SLC40A1* IVS3+10 del gtt mutation, with normal liver tissue as control. a = Normal liver tissue, 40× magnification, b = the HH patient with *SLC40A1* IVS3+10 del gtt mutation, 40× magnification. HH = hereditary hemochromatosis, RT-PCR = reverse transcriptase-polymerase chain reaction.

Table 1**Comparison of phenotypic profiles between type 4A and type 4B hereditary hemochromatosis.**

	Serum iron and transferrin saturation	Serum ferritin	Iron deposition on liver biopsy	MRI	Response to phlebotomy	Prognosis
Type 4A (classical)	Normal or mild elevated	Usually much higher than type 4B	Predominate in the Kupffer cells	Iron overload prevails in the spleen ("black spleen and grey liver")	Low tolerance with risk of anemia	Relative benign
Type 4B (nonclassical)	Elevated markedly	Mild, moderate to severe, vary with liver and body iron overload	Predominate in the hepatocytes, lack of iron in Kupffer cells if liver biopsy performed at early stages	Diffuse hepatic iron excess without splenic iron ("black liver and white spleen")	Tolerance, strong efficient	Chronic iron overload progressively damages the hepatocytes leading to hepatomegaly, progressive fibrosis and cirrhosis with the risk of hepatocellular carcinoma

MRI = magnetic resonance imaging.

the *SLC40A1* IVS3+10 del gtt mutation may lead to abnormal splicing of mRNA.

2.4. Immunohistochemical analysis of ferroportin

Sections (4 μm thick) were cut for immunohistochemistry (IHC) analysis. After deparaffinization of the slides, endogenous peroxidase activity was blocked with 0.3% H₂O₂, in methanol for 30 minutes. Antigen retrieval was carried out in antigen unmasking solution (H-3300, Vector) with microwaving for 15 minutes, keeping the solution boiling and then treating with 5% skimmed milk in phosphate buffer saline-0.1% albumin from bovine serum for at least 1 hour at room temperature to block nonspecific staining. Immunohistochemical staining was performed using antibodies against ferroportin (NBP1-21502, Novus) at a dilution of 1:800 at 4°C overnight. Secondary antibody (MP-7401, Vector) was used at 37°C for 1 hour, and visualization of antigen-antibody reactions were achieved with 3,3'-diaminobenzidine (DAB, Vector kit SK-4100).

Increased staining of ferroportin was shown in the cell membranes in the liver tissue with the *SLC40A1* IVS3+10 del gtt compared with that of the normal liver tissue, suggesting the increased membrane localization of ferroportin (Fig. 4B).

3. Discussion

Currently, 4 major categories of hereditary hemochromatosis have been established, including *HFE*-related or type 1 hemochromatosis (which is common in Caucasians), type 2 (A and B) hemochromatosis with mutation in *HJV* or *HAMP*, type 3 hemochromatosis with mutation in *TFR2*, and type 4 hemochromatosis (ferroportin disease) with mutation in *SLC40A1*.^[6] In this report, we describe the clinical and laboratory features of a case diagnosed with HH, who has a previously unrecognized ferroportin mutation, specifically, *SLC40A1*: IVS3+10 del gtt, which is a classical splicing mutation. To our knowledge, more than 30 case reports of ferroportin disease had been reported,^[7] but all the mutations reported in those reports were missense mutation without functional analysis, so the biological consequence of the identified mutations remains to be explored. However, the case in the present study is the first report to identify a classical splicing mutation in the *SLC40A1* gene in type 4B hemochromatosis.

Ferroportin disease (type 4 HH) is due to pathogenic mutations in the *SLC40A1* gene, which encodes a main iron export protein called "ferroportin1/IREG1/MTP1."^[8] By altering the physicochemical properties of this protein, these mutations cause iron overload via an intracellular retention mechanism.^[9] According to the different phenotypes, ferroportin disease is divided into 2 types: type 4A (classical ferroportin disease) and type 4B (nonclassical ferroportin disease).^[9] The phenotypic features of type 4A and type 4B HH are summarized in Table 1.^[7-9] It is assumed that the phenotypes of patients with *SLC40A1* mutations vary depending on the mutation.^[10] Mutations leading to ferroportin that is either not present normally at the cell surface or has defective iron export activity (loss of function) are associated with iron deposition in Kupffer cells and low transferrin saturation,^[10] and define type 4A. Those mutated ferroportin that cannot be internalized after hepcidin binding (gain of function) are associated with iron deposition in hepatocytes and elevated transferrin saturation,^[10] and define type 4B. A systemic meta-analysis of 176 individuals reported that *SLC40A1* mutations, namely, V162del, D157N, D181V, G80V, Q182H, and R489K, may impair ferroportin's iron export function, and are common mutations for type 4A HH. Conversely, the mutations Y64N, V72F, and Y501C confer hepcidin resistance and are common mutations for type 4B HH.^[7]

Chen et al.^[11] reported the first type 4B HH case in the Chinese Han population. They found a novel heterozygous mutation (p. Cys326Phe) in the *SLC40A1* gene, which results in hepcidin resistance, although this finding needs in vitro confirmation. Our case here is the first case report of type 4B HH associated with the identified IVS3+10 del gtt mutation in the *SLC40A1* gene. The typical splicing mutation, IVS3+10 del gtt, is located in intron 3 of the *SLC40A1* gene, 9 bases away from the end base of exon 3. Subsequently, this mutation may lead to a splicing error that results in incorrect assembly of ferroportin protein and a consequent change in function. In a recent study, a single heterozygous *MSX1* IVS1+5G/A mutation was identified in a case affected with tooth agenesis. Although RNA sequencing showed correct joining of exon 1 and exon 2 sequences, a substantial reduction of the levels of *MSX1* mRNA was observed, indicating that the novel variant may reduce efficiency of the splicing of pre-mRNA, and thus lead to the decrease of mRNA level.^[12] Previous studies have demonstrated the reduced efficiency of pre-mRNA splicing in other genes containing the

intronic mutation downstream of the consensus donor site.^[13] In the above study, primer sets targeting exon 1 and 2 sequences were used to assess cDNA replicates. Compared with unaffected individuals, significantly reduced *MSX1* mRNA expression was observed in the proband. Similarly, in the present study, primer sets targeting exon 3 and 4 sequences were used to assess cDNA replicates, and the results showed significantly reduced *SLC40A1* mRNA expression compared with that of the controls, suggesting the *SLC40A1* IVS3+10 del gtt may lead to abnormal splicing of mRNA.

In addition, the IHC analysis of ferroportin showed increased staining of ferroportin in the cell membranes in the liver tissue with the *SLC40A1* IVS3+10 del gtt, compared with that of the normal liver tissue. The increased membrane localization of ferroportin, one of the characteristics of type 4B HH, may result from the abnormal *SLC40A1* transcripts, of which the encoded mutated ferroportin cannot be internalized after hepcidin binding.

This patient presented with abnormal liver function, diabetes mellitus, hyperferritinemia, and high transferrin saturation, with compound iron overload in parenchymal cells and Kupffer cells, periportal fibrosis on liver biopsy, and was heterozygous for a novel mutation in the *SLC40A1* gene. Therefore, the final diagnosis of the patient was type 4B (nonclassical) hereditary hemochromatosis.

4. Conclusions

In conclusion, we have identified the IVS3+10 del gtt mutation of ferroportin 1 in a Chinese individual. This mutation is associated with high transferrin saturation, parenchymal iron loading, and advanced fibrosis. This case is the first report to identify a classical splicing mutation in the *SLC40A1* gene and emphasizes the importance of non-*HFE* mutations in hereditary hemochromatosis in Asian countries.

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