Int J Clin Exp Pathol 2016;9(3):3313-3321
www.ijcep.com /ISSN:1936-2625/IJCEP0020975

Original Article

Association between hepatic iron deposition, and serum bilirulin levels, mutations of UGT1A1 and HFE gene in patients with hereditary unconjugated hyperbilirubinemia

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Received December 1, 2015; Accepted February 12, 2016; Epub March 1, 2016; Published March 15, 2016

Abstract: Histological findings of liver in patients with hereditary unconjugated hyperbilirubinemia (HUH) are generally mild. But Iron deposition in liver shown in some patients may indicate iron overload which can be contributing factor to additional complications. Mechanism of increased iron deposition is unclear. We studied the features of pathological changes, bilirubin levels, mutations of UGT1A1 and HFE gene among patients with iron deposition and compared with patients without iron deposition. Liver biopsies from 59 patients with HUH were stained with HE, Masson's trichrome, Reticulin and Perls' Prussian blue. Ishak and Deugnier scoring systems were used to assess inflammatory activity, the extent of fibrosis and distribution of iron deposition, respectively. Serum were used for evaluation of bilirubin levels, DNA amplification and sequencing were performed to detect mutations of genes. Of the 59 patients, 20 patients (33.9%) had iron deposition in the liver. The pattern of iron deposition is similar to the HFE-related hereditary hemochromatosis (HH), mainly in periportal hepatocytes, and the Deugnier score of parenchymal iron deposition was significantly higher than mesenchymal score ($P<0.001$). Total serum bilirubin levels ($P=0.009$), unconjugated bilirubin levels ($P=0.024$) and the frequency of heterozygous mutations in UGT1A1 gene ($P=0.001$) were also significantly higher in patients with iron deposition. Ten of the twelve patients (83.3%) with iron deposition who underwent HFE testing had an intronic variant in intron 2 (IVS2+4T→C). Iron deposition in liver biopsies of patients with HUH is related to increased bilirubin levels and mutations of UGT1A1 gene and HFE gene.

Keywords: Crigler-Najjar syndrome, Gilbert's syndrome, iron overload, liver pathology

Introduction

Hereditary unconjugated hyperbilirubinemia is an autosomal dominant hereditary condition with incomplete penetrance that is characterized by intermittent unconjugated hyperbilirubinemia and associated with UGT1A1 gene polymorphism which is responsible for bilirubin conjugation. Variants in UGT1A1, either in an exon or the promoter, may result in UGT1A1 enzyme deficiency in patients with hereditary unconjugated hyperbilirubinemia, including Gilbert's syndrome (GS) and Crigler-Najjar syndrome (CNS).

GS is the most commonly inherited disorder of bilirubin metabolism, affecting 3-12% of the general population, mainly characterized by intermittent unconjugated hyperbilirubinemia in the absence of hepatocellular disease or hemolysis, which can become clinically apparent during fasting, physical exercise, stress, or menstruation [1, 2]. CNS forms are classified into two types based on serum total bilirubin concentrations (STBC): the severe type (CNS-I) is defined by high levels of STBC (342-684 μmol/L), while the milder type, namely CNS-II, is defined by lower level of STBC ranging from 103 to 342 μmol/L [3].

Histological findings of liver in patients with hereditary unconjugated hyperbilirubinemia are generally mild, with a slight centrilobular accumulation of pigments that have lipofuscin-like

Table 1. Demographics of patients with or without iron deposition

	Patients with iron deposition (n=20)	Patients without iron deposition (n=39)	All patients (n=59)
Gender			
Male	18 (30.5%)[a]	25 (42.4%)	43 (72.9%)[b]
Female	2 (3.4%)	14 (23.7%)	16 (27.1%)
Age			
11-20	1 (1.7%)	9 (15.2%)	10 (16.9%)
21-30	6 (10.2%)	11 (18.6%)	17 (28.8%)
31-40	7 (11.9%)	9 (15.2%)	16 (27.1%)
41-50	3 (5.2%)	10 (16.9%)	13 (22.1%)
51-60	2 (3.4%)	0	2 (3.4%)
61-70	1 (1.7%)	0	1 (1.7%)

[a]*P*-value <0.01 compared to female with iron deposition. [b]*P*-value <0.05 compared to female.

properties [4]. The morphological presentation of GS and CNS is considered non-specific; therefore, little attention has been given to other pathological changes it might be associated with, one of which is iron deposition in liver. Our early study has found that mild or moderate iron overload probably is not rare in these patients. Sometimes, liver iron deposition is found in liver biopsies of patients without any clinical symptoms of iron overload. This finding should be included in the pathology report and investigated further because iron overload, even mild, is a suspected contributing factor for various conditions, for example, it not only promotes fibrogenesis that can result in accelerated progression to liver cirrhosis but also increases the risk of insulin-resistance syndrome, diabetes and hepatocellular carcinogenesis.

While the mechanism of liver iron deposition in patients with GS and CNS remains unclear, it is important to pay attention to these pathological changes given the associated complications. Therefore, to explore the pathogenesis of iron accumulation, we described the pattern of liver iron deposition in patients with GS and CNS, and also investigated the association between serum bilirubin levels, UGT1A1 gene mutations and liver iron deposition. Because the most common primary genetic disorder of iron metabolism is HFE gene-associated HH [5], we also detected HFE gene mutations in some of patients with iron deposition.

Materials and methods

Study subjects

A total of 59 patients with hereditary unconjugated hyperbilirubinemia that had serum total bilirubin level ≥17.1 μmol/L, normal levels of liver enzymes and no symptoms and signs indicative of other hepatobiliary disease as well as no evidence of hemolysis were enrolled in Beijing Ditan Hospital, Beijing, China, between 2009 and 2014. Serum samples were collected after an overnight fast, and all the biochemical tests were performed in the same laboratory with standardized methods. No abnormalities were found in abdominal Ultrasound imaging. No treatment was administered. Demographic and clinical data were obtained from patients' electronic medical records.

All patients underwent percutaneous needle biopsy of the liver (1.4 mm in diameter), and classified into two types based on liver iron deposition.

A written informed consent from all participants was obtained prior to study participations. The study protocol was approved by the Ethics Committee of Beijing Ditan Hospital, Capital Medical University.

Histopathological measurements

Paraffin-embedded blocks of liver specimens were sectioned and stained with hematoxylin-eosin (HE), Masson's trichrome, and Reticulin. Perls' Prussian blue stain was used to evaluate the degree and cellular distribution of hepatic iron deposition. All sections were evaluated by the same pathologist. Tissue samples were also graded for inflammatory activity and staged for fibrosis using the Ishak scoring systems [6]. Iron deposition in liver specimens were evaluated according to the Deugnier scoring systems [7].

DNA extraction and genetic analysis

Peripheral blood samples were obtained and stored in tubes containing ethylenediaminetetraacetic acid (EDTA), and separated by centrifu-

Table 2. Biochemical parameters of patients with or without liver iron deposition

Biochemical parameters	Patients with iron deposition (n=20)	Patients without iron deposition (n=39)	Normal range	*P* value
ALT, U/L	24.44±15.50	23.15±16.26	9-50	0.773
AST, U/L	19.64±5.79	20.46±8.20	15-40	0.695
TBIL, μmol/L	96.68±46.37	64.96±25.04[a]	<18.8	0.009
IBIL, μmol/L	79.05±40.43	55.04±26.05[b]	<12	0.024
ALP, U/L	65.58±28.38	88.01±48.74	45-125	0.073
GGT, U/L	33.69±44.45	26.76±35.75	10-60	0.541
TP, g/L	73.54±6.69	73.16±6.30	65-85	0.836
ALB, g/L	46.23±2.64	46.41±4.47	40-55	0.845
GLO, g/L	27.26±5.19	27.09±3.90	20-40	0.893

ALT: alanine aminotransferase, AST: aspartate aminotransferase, ALP: alkaline phosphatase, GGT: Gamma-glutamyl transferase, TP, Total protein, ALB, Albumen, GLO, Globulin. Data are expressed as mean ± SD. *P* values were calculated using the t-test. [a]*P*-value <0.01 compared to patients with iron deposition. [b]*P*-value <0.05 compared to patients with iron deposition.

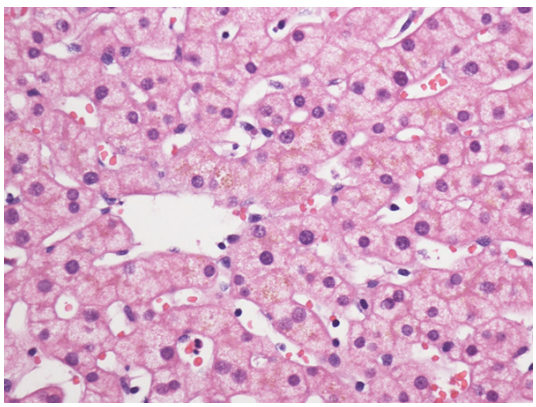

Figure 1. lipofuscin-like pigment in perivenular hepatocytes HE × 40.

gation at 2000 rpm at 4°C for 15 min. Genomic DNA was isolated from frozen EDTA-anticoagulated blood samples according to the membrane-based QIAamp DNA extraction protocol (QIAGEN, Hilden, Germany).

The promoter, 5 exons, flanking intronic regions, and the phenobarbital response enhancer module of UGT1A1 were amplified with PCR. Single nucleotide polymorphisms (SNPs) of UGT1A1 and UGT1A1 genes were searched using the National Center for Biotechnology Information (NCBI) database. Primers were designed using online primer design software Primer 3 according to the published UGT1A1 sequence. The reaction mixture contained 200 ng of genomic DNA, 1 × reaction buffer, 0.2 mmol/L dNTPs, 1.5 mmol /L $Mgcl_2$, 1U of Taq DNA polymerase, 0.4 nmol/L of each primer in a final volume of 50 μl. PCR conditions were as follows: initial denaturation at 94°C for 2 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and elongation at 72°C for 30 s. Fluorescent PCR products were analyzed with an ABI Prism 310 Genetic Analyzer (Applied Biosystem, Foster City, CA, USA) using the reagents according to the manufacturer's protocol.

To amplify coding sequences and exon-intron boundaries of the HFE, HAMP, TFR2, SLC40A1 and HJV genes in 12 patients with liver iron deposition that approval were obtained, primers for PCR were synthesized as previously described [8-12]. Amplification and direct sequencing to detect mutations of these genes were performed.

Statistical analysis

All statistical tests were performed using SPSS (IBM statistics, Version 19.0, SPSS, Chicago, USA). Independent student t-test was used to determine differences between patients with or without hepatic iron deposition. Categorical variables were analyzed using the χ^2 test. Continuous data were expressed as mean ± SD, and a *P*-value <0.05 was considered to be statistically significant.

Results

Patient characteristics

Among 59 patients with hereditary unconjugated hyperbilirubinemia finally enrolled in this study, 20 of them had iron deposition in the liver, accounting for 33.9% of the cohort, in comparison to 66.1% (39 patients) without hepatic iron deposition. The patients were predominantly male (72.9%), especially in the 20 individuals with iron deposition (90%). In this

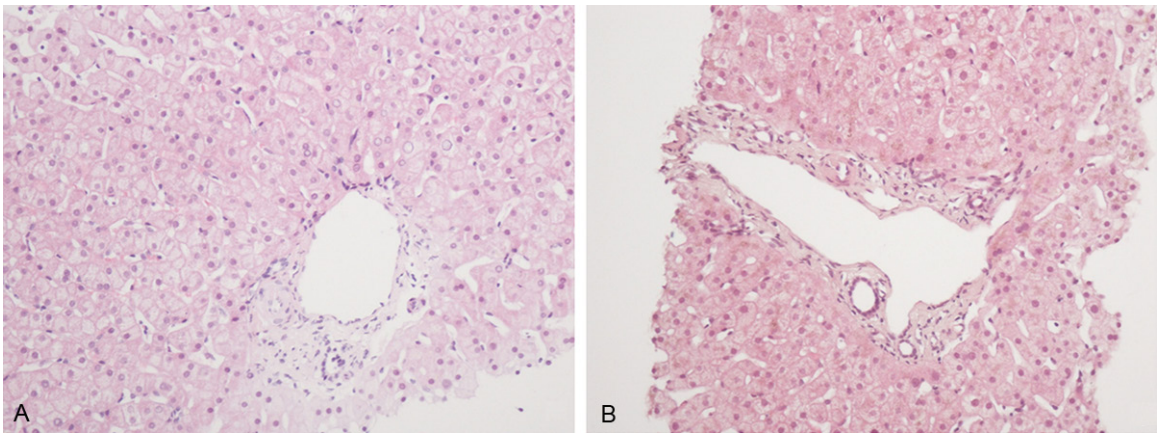

Figure 2. A few or minimal lymphocytes infiltration in portal tracts (A. No iron deposition. B. Iron deposition in periportal hepatocytes) HE × 20.

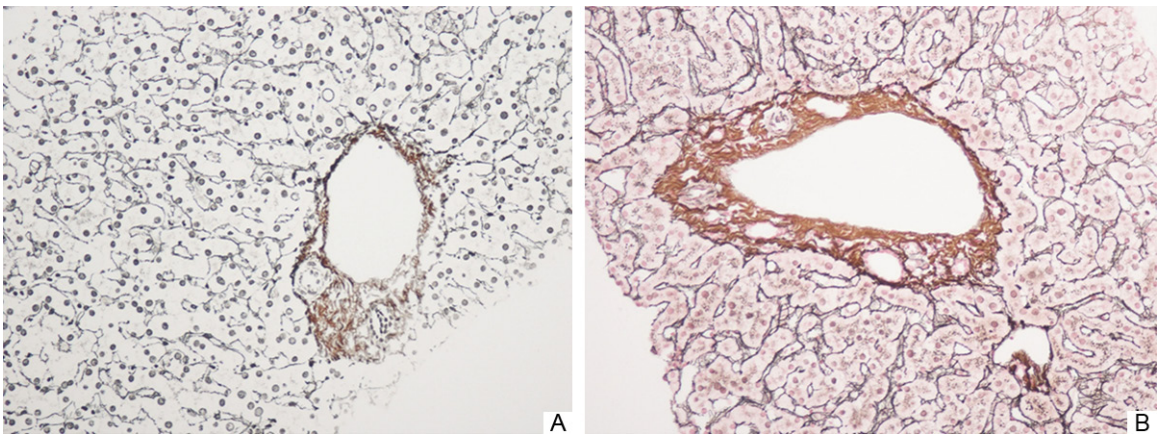

Figure 3. Fibrosis mainly in a portal distribution (A. No iron deposition. B. Iron deposition in periportal hepatocytes) Reculin × 20.

study, the onset age of unconjugated hyperbilirubinemia ranged from 13 to 64 years. The demographic information was shown in **Table 1**.

Clinical characteristics

Serum total bilirubin levels (TBIL) and unconjugated bilirubin levels were beyond normal range in all 59 patients, including 44 cases of GS and 15cases of CNS-II, no CNS-I. The group of patients with liver iron deposition was comprised of 10 GS and 10 CNS-II. Furthermore, TBIL and IBIL of the patients with liver iron deposition were significantly higher than those of patients without iron deposition (*P* values =0.009 and 0.024, respectively).

Other measurements such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), Gamma-glutamyl transferase (GGT), Total protein (TP), Albumen (ALB), Globulin (GLO) were all in normal range, and had no difference between the two groups. The data are shown in **Table 2**.

Histology

Based on liver biopsies of all patients, the liver structure was normal with increasing lipofuscin-like pigments in perivenular and midzonal hepatocytes (**Figure 1**). Only a few or no lymphocytes infiltrated in parts of the portal tract (**Figure 2**). In some patients, liver damage was characterized by the appearance of collagen deposition or fibrosis, mainly in a portal distribution (**Figure 3**). The Ishak scores for Inflammation and Fibrosis in 59 patients were 1.57±0.62 and 0.86±0.44, respectively.

Table 3. Deugnier and Ishak scores from liver biopsy

	Deugnier scores		Ishak scores	
	Hepatocytic iron deposition	Sinusoidal and portal iron deposition	Inflammation grading	Fibrosis staging
Patients with iron deposition (n=20)	10.65±6.12	0.70±1.81[a]	1.50±0.69	0.98±0.41
Patients without iron deposition (n=39)	NA	NA	1.61±0.59	0.80±0.45
P value	NA	<0.001	0.536	0.148

[a]*P*-value <0.001 compared to Deugnier scores of hepatocytic iron deposition.

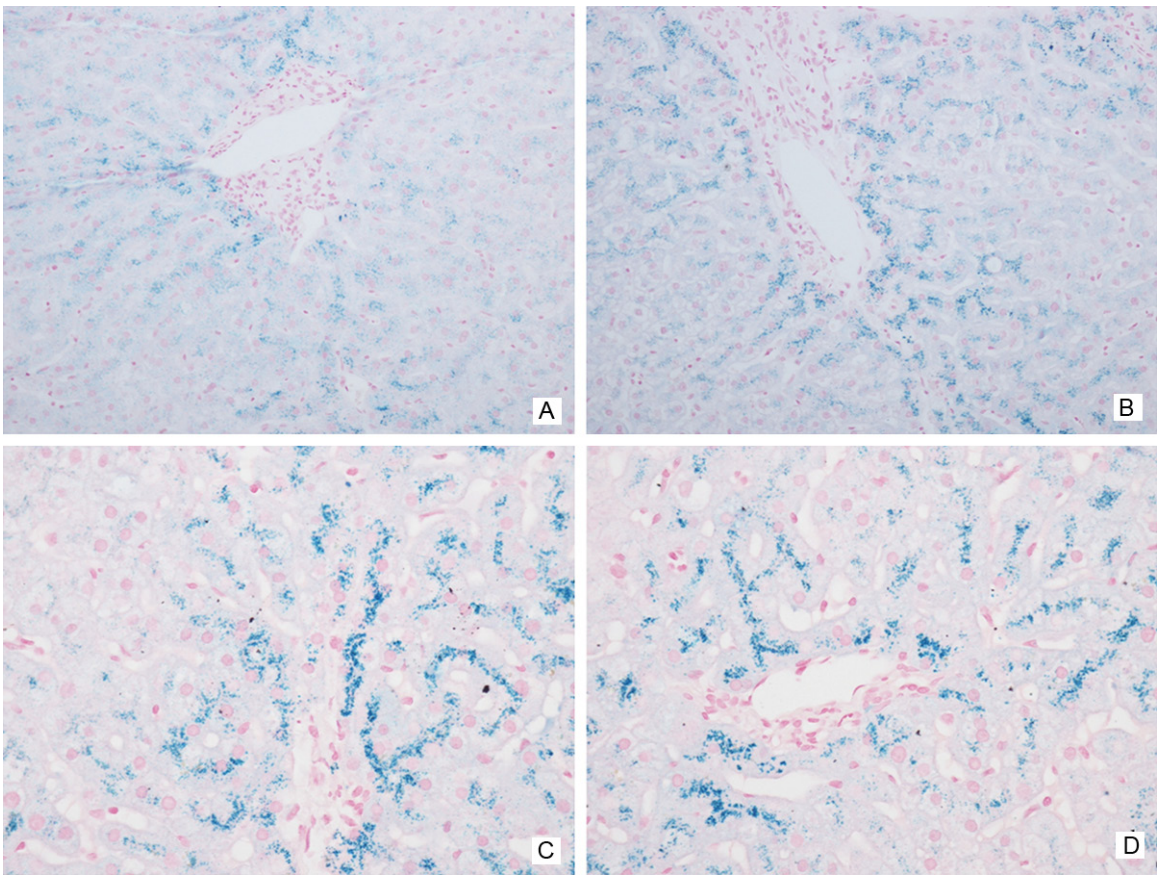

Figure 4. Iron deposits mainly in periportal hepatocytes, distributing according to a decreasing gradient from periportal to centrolobular areas (A and B). Perls' Prussian blue × 20. Iron remains at the biliary pole of hepatocytes (C and D). Perls' Prussian blue × 40.

On liver biopsies of patients with hepatic iron deposition, the Ishak score for Inflammation was 1.50±0.69, slightly less than patients without iron deposition (P=0.536). In contrast, the Ishak score for Fibrosis was 0.98±0.41, mildly higher than patients without liver iron deposition (P=0.148). Ishak scores between the two groups were not statistically significant, which may be due to insufficient number of patients in our study. The Ishak scores are shown in **Table 3**.

Hepatic iron deposition

Iron is stored mainly in periportal hepatocytes like what is seen in HFE C282Y homozygotes hereditary hemochromatosis (HH) with relatively sparing of sinusoidal cells and portal macrophages. The Deugnier scores of parenchymal and mesenchymal iron deposition were 10.65 ±6.12 and 0.70±1.81, respectively. Parenchymal score was significantly higher than mesenchymal score (**Table 3**). Iron was distributed

Table 4. Incidence of UGT1A1 variants by groups

	Mutations in group with iron deposition (n=36)		Mutations in group without iron deposition (n=68)	
	Homozygous	Heterozygous	Homozygous	Heterozygous
PBREM	2 (5.6%)	11 (30.6%)	12 (17.6%)	11 (16.2%)
A(TA)nTAA	2 (5.6%)	9 (25%)	9 (13.2%)	10 (14.7%)
p.G71R	1 (2.8%)	11 (30.6%)	2 (2.9%)	8 (11.8%)
p.P229Q	0	0	1 (1.5%)	5 (7.4%)
p.P364L	0	0	0	2 (2.9%)
p.Y486D	0	0	6 (8.8%)	2 (2.9%)
All mutations	5 (13.9%)	31 (86.1%)[a]	30 (44.1%)	38 (55.9%)

UGT1A1, uridine diphosphate-glucuronyl Transferase 1A1; PBREM, phenobarbital response enhancing motif; [a]*P*-value <0.01 compared to heterozygous without iron deposition.

according to a decreasing gradient from periportal to centrolobular areas (**Figure 4**) and remains could be found at the biliary pole of hepatocytes (**Figure 4**).

UGT1A1 variants in all patients

A total of 6 types of variants were detected in two groups, including c.-3279T>G in PBREM, A (TA) 7TAA, p.G71R, p.P229Q, p.P364L, p. Y486D. The first three mutations are most common in these patients. The frequencies of these variants were 34.6%, 28.8%, 21.2%, 5.8%, 1.9% and 7.7%, respectively. There was no significant difference between the two groups. But in patients with iron deposition, only the first three mutations were detected. There were 36 mutations in 20 patients with iron deposition, and 68 mutations in 39 patients without iron deposition. The frequency of heterozygous mutations in patients with iron deposition was noticeably higher than that in patients without iron deposition (86.1% vs 55.9%, respectively, P<0.01, **Table 4**).

HFE variants in patients with iron deposition

We also detected mutations of the HFE, HAMP, TFR2, SLC40A1 and HJV genes in 12 patients with iron deposition. None of the individuals who underwent HFE testing was a C282Y homozygote or compound heterozygote. Only 3 patients was heterozygous for the H63D mutation in exons 2 (Supplementary Figure 1), accounting for 25% (3/12). Among them 1 patient also had an intronic sequence variant in intron 2 (IVS2+4T→C), plus 9 other patients who also had the intronic variant in intron 2 (IVS2+4T→C), there were 10 patients with intronic variant in total, accounting for 83.3% (10/12). Of them 6 patients were homozygous for the intronic variant, while 4 were heterozygous (Supplementary Figure 2). No other mutations were found.

Discussion

Iron is essential for erythropoiesis and other metabolic processes, and the body iron levels are precisely regulated under normal physiologic conditions. When the regulation is disrupted by pathological factors, absorption of dietary iron will increase inappropriately leading to iron deposition in many organs, primarily in the liver. Many chronic liver diseases can cause iron deposition in liver as well, such as hereditary haemochromatosis, chronic viral hepatitis, and nonalcoholic fatty liver disease. In this study, we found that 33.9% patients with hereditary unconjugated hyperbilirubinemia had iron deposition in the liver that had not been reported, and 90% of these patients were male. This finding is consistent with early report that males had a higher incidence of iron deposition. The gender difference may be due to menstrual blood loss and maternal iron loss during pregnancy that might have a “protective” effect for women [13]. Iron deposition in liver may affect parenchymal cells (mainly hepatocytes) and mesenchymal cells (mainly Kupffer cells, endothelial cells, macrophages within portal tracts). In our study iron deposition in the liver of patients with hereditary unconjugated hyperbilirubinemia was predominantly in parenchymal pattern, similar to what has been seen in HFE C282Y HH, which is characterized by iron deposition within hepatocytes.

It has been reported that chronic liver damage from iron deposition was non-inflammatory, or minimal inflammatory cell infiltration [14]. Our results are consistent with these findings. The Ishak scores of Inflammation grading in all patients were very low. Another study has suggested that the characteristics of chronic liver damage by iron deposition was the appearance of fibrosis, initially in a periportal distribution, eventually bridging between portal tracts and central veins [14]. Our study had the similar

results, showing that main damage in the liver of patients with iron deposition was portal fibrosis, and the Ishak score of Fibrosis staging is mildly higher than patients without iron deposition. The pathogenesis of liver damage is mainly related to the oxidative damage induced by iron followed by impairment of membrane-dependent functions of mitochondria, microsomes, and lysosomes [15]. Iron-induced membrane lipid peroxidation occurs in hepatocytes and causes hepatocellular injury or death. Also, Kupffer cells become activated by products released from injured iron-loaded hepatocytes and produce profibrogenic cytokines. These cytokines can stimulate hepatic stellate cells to synthesize increased amounts of collagen, thereby leading to pathologic fibrosis [16].

Our study also demonstrated that serum bilirubin levels among patients with iron deposition were significantly higher than those patients without iron deposition. Previous data have demonstrated the anti-inflammatory, antioxidant and lipid-lowering effects of bilirubin [17, 18]. Inflammatory stimuli induce expression of hepcidin [19] which is a negative regulator of cellular iron export by binding to its receptor, ferroportin (Fpn), that can be found in macrophages and on the basolateral surface of enterocytes. So when the process of inflammation is suppressed by bilirubin, hepcidin expression decreases and subsequent upregulation of iron levels occurs. So elevated levels of bilirubin might promote iron loading by decreasing oxidative stress and inhibiting hepcidin expression signaling due to reduced inflammatory activity [20]. These were consistent with the findings from liver biopsies in our study, that the Ishak score of Inflammation grading of patients with iron deposition was mildly lower than patients without iron deposition.

UGT1A1 gene encodes the main glucuronidation enzyme for bilirubin [21]. Up to now, more than 100 mutations of the UGT1A1 gene have been reported in the Human Gene Mutation Database (www.hgmd.cf.ac.uk). In our study, the most frequent variant of GS in Chinese population was the c.-3279T>G in PBREM. The frequency of c.-3279T>G in PBREM, A (TA) 7TAA, c.211G > A was 38.4%, 31.5%, 13.7%, respectively. But in 15 cases of CNS-II in our study, the most frequent mutation was the p.G71R variant in exon 1, accounting for 38.7%. Also, other variants in coding regions such as UGT1A1*7 (p.Y486D), UGT1A1*27 (p.P229Q) were detected in our study. A (TA) 7TAA is the most common genetic variant in Caucasians and African Americans, most of them with GS are homozygous A (TA) 7TAA. However, this variant is much less frequent in East Asians [22]. In Japanese population, the most frequent genetic mutation associated with GS is the c.211G > A variant occurring in exon 1 [23]. It was interesting to find that the frequency of heterozygous mutations of UGT1A1 gene in patients with iron deposition which had higher bilirubin levels was significantly higher than that in patients without iron deposition. So we hypothesize that heterozygous mutations of UGT1A1 gene may be associated with higher levels of bilirubin. Similar results were also reported by Skierka JM et al. [24]. Their data demonstrated that individuals who were heterozygous for the A (TA) 7TAA allele had higher levels of bilirubin compared with those homozygous for A (TA) 7TAA. It is likely that the heterozygous individuals had other confounding factors leading to their Hyperbilirubinemia.

HFE-related HH is the most common type of HH in Europeans, approximately 85%-90% of HH patients are homozygous for the C282Y mutation in the HFE gene [25]. A small minority have compound heterozygotes, C282Y/H63D or C282Y/S65C [13]. And it has been reported that the H63D mutation (homozygous or heterozygous) was not associated with significant iron overload [26]. Unlike European countries, in Asia-Pacific regions only a small percentage of HH was the C282Y mutation [27]. Same findings were identified in our study, all Chinese patients, where C282Y mutation was absent in all patients, so were HJV, TFR2, SLC40A1, HAMP genes. On the other hand, we found heterozygous H63D mutation in 25% and intronic variant in intron 2(IVS2+4T→C) in 83.3% of 12 patients with iron deposition in hepatocytes. The location of variant in intron 2 is a restriction enzyme recognition site where *Rsa*I polymorphism has been reported [28]. The mutation in intron may affect gene splicing to produce a mild phenotypic effect or linkage disequilibrium with another disease-related mutation. The frequency of the mutant C-allele was 25% in 24 control individuals, compared with 38% in the 13 HH patients. Of 12 patients with iron deposition and underwent HFE test-

ing, 83.3% (10/12) had intronic variant in our study. The apparent excess of the mutated allele in the group of patients with iron deposition may be due to chance [28]. Further research is needed to investigate the relationship between HFE intron 2 variant and UGT1A1 gene.

In conclusion, we reported the pattern of iron deposition in patients with hereditary unconjugated hyperbilirubinemia which was similar to the HFE-related hereditary hemochromatosis, mainly in periportal hepatocytes. Total serum bilirubin levels, unconjugated bilirubin levels and the frequency of heterozygous mutations in UGT1A1 gene of patients with iron deposition were all higher than the group of patients without iron deposition and most patients who had iron deposition and underwent HFE testing also had an intronic variant in intron 2 of HFE(IVS2+4T→C). The relationship between the intronic variant and UGT1A1 is not clear. The intronic variant may be a confounding factor for the effect of heterozygotes UGT1A1 gene mutation on increasing serum bilirubin levels which may further leads to iron deposition, or as a gene mutation of iron metabolism that directly induce iron deposition in the liver. To which further exploration is warranted.

Disclosure of conflict of interest

None.

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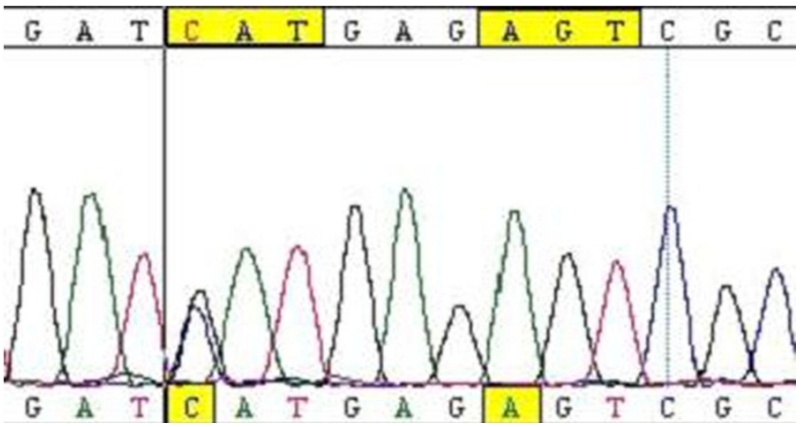
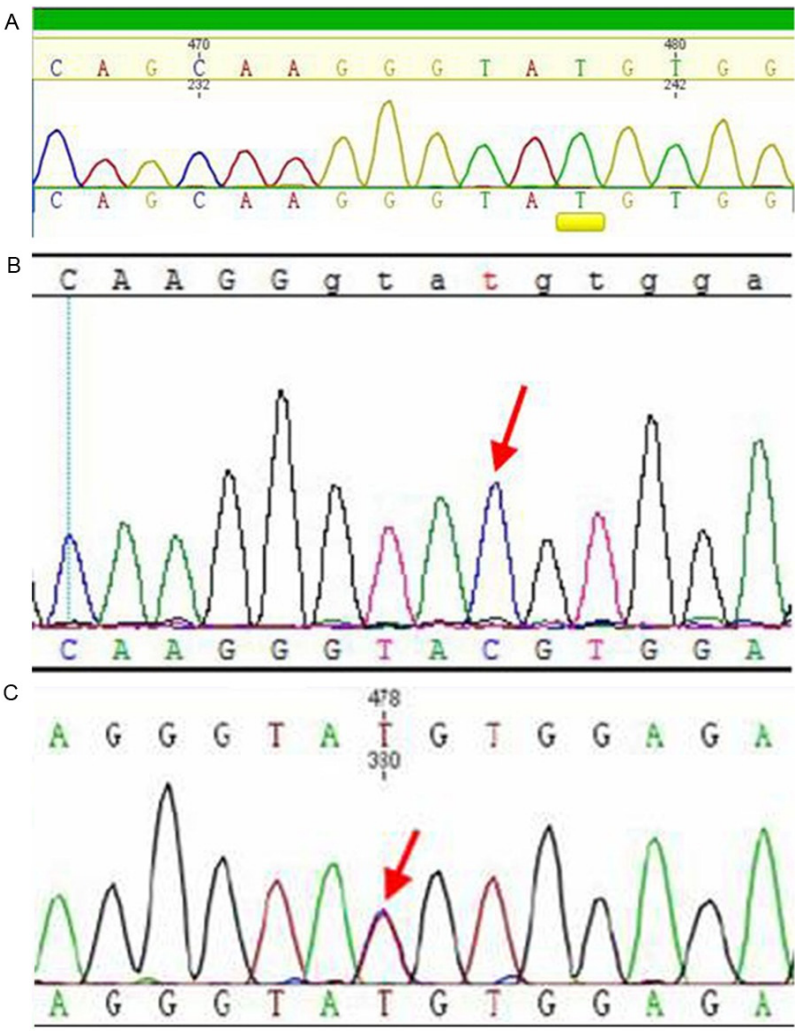


Supplementary Figure 1. Heterozygous(C/G) for H63D mutation.



Supplementary Figure 2. Mutations of IVS2+4T→C (A. Wild type (T/T). B. Homozygous (C/C). C. Heterozygous (T/C)).