Correlation of genotype and phenotype in 32 patients with hereditary hemochromatosis in China

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Abstract

Background: Hereditary hemochromatosis (HH) is widely recognized and clinical manifestations of hemochromatosis-related (HFE-related) HH is well studied in European populations. Less is known about the clinical and laboratory characteristics of non-HFE related HH in Asian population. We aimed to explore the relationship between genotype and clinical phenotype in Chinese patients with non-HFE related hereditary hemochromatosis.

Methods: Peripheral blood samples and clinical data of patients with primary iron overload were collected from the China Registry of Genetic/Metabolic Liver Diseases. Sanger sequencing was performed in cases with primary iron overload, for 5 known HH related genes (HFE, HJV, HAMP, TFR2 and SLC40A1) and 2 novel iron homeostasis-related genes (DENND3 and SUGP2). The correlation of genotype and clinical phenotype in these patients was analyzed.

Results: Of the 32 patients with primary iron overload (23 were males and 9 were females), non-HFE variants were detected in 31 (31/32, 97%), including 8 pathogenic variants in HJV, 7 pathogenic variants in SLC40A1, 8 likely pathogenic variants in SUGP2 and 5 likely pathogenic variants in DENND3 cases. Among these 31 cases, 4 cases harbored homozygous variants, 2 cases harbored homozygous + heterozygous variants, 19 cases harbored heterozygous or combined heterozygous variants, and 6 cases harbored no any damaging variants. None of investigated cases carried damaging HAMP and TFR2 variants were found. 8 cases were classified as type 2A HH and 6 cases as type 4 HH, 10 cases as non-classical genotype, and 6 cases had no pathogenic variants from 31 cases. During the statistical analysis, we excluded one case (SLC40A1 IVS3 +10delGTT+SUGP2 p. R639Q(homo)) with difficulty in grouping due to combined damaging variants. Cases with type 2A HH have an earlier age at diagnosis (p = 0.007). The iron index of cases in type 2A HH and type 4 HH was higher than that in other groups (p = 0.01). Arthropathy was relatively rare in all groups. None of cases with type 2A HH developed cirrhosis. Cirrhosis and diabetes are more prevalent in type 4 HH. The incidence of cirrhosis (p = 0.01), cardiac involvement (p = 0.042), diabetes (p = 0.035) and hypogonadism (p = 0.02) was statistically significant in the four groups. However, due to the limited sample size, the pairwise comparison showed no significant difference.

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**Introduction**

Hereditary haemochromatosis (HH) is an iron-storage disease, caused by mutations in genes involved in the regulation of iron homeostasis, resulting in excessive absorption and toxic accumulation of iron in the liver, pancreas, skin, heart, joints, and anterior pituitary gland [1]. In untreated individuals, iron overload can lead to liver fibrosis/cirrhosis, diabetes, skin pigmentation, heart disease, bone and joint disease, and hypogonadism. Hereditary haemochromatosis is associated with malignancies, particularly hepatocellular carcinoma [2]. Early recognition, diagnosis and treatment for haemochromatosis can reduce iron deposition and prevent disease progression [3, 4].

There are 4 main types of HH that have been categorized based on which proteins involved in iron homeostasis are affected [5]. Type 1 HH is the most common form of HH in Caucasian populations, which is caused by homozygous p. C282Y or compound heterozygous p.C282Y/H63D mutations in HFE gene [6]. Type 2A, type 2B, type 3, and type 4 are associated with pathogenic defects in the hemojuvelin (HJV), hepcidin (HAMP), transferrin receptor 2 (TFR2) and ferroportin (SLC40A1) genes, respectively [7]. Mutations in the HFE, HJV, HAMP and TFR2 genes result in an autosomal recessive form of HH, whereas mutation in the SLC40A1 gene results in an autosomal dominant form of HH [7].

The majority of HH cases are related to non-HFE genes in Asian countries [8, 9]. In addition, some novel gene variants related to the regulation of iron homeostasis have been identified in Chinese HH patients. In our previous study, SUGP2 p. R639Q, BMP4 p. R269Q, and DENND3 p. L708V were first identified in HH patients [10].

However, less is known about the clinical features and genetic correlation of non-HFE HH in Asian populations. Therefore, in the present study, we investigated the genetic characteristics and relationship between genotype and phenotype of non-HFE HH in a cohort of patients with HH in China.

**Methods**

**Patients**

Patients with iron overload were enrolled from the China Registry of Genetic/Metabolic Liver Diseases (CR-GMLD, Clinical trials. gov: NCT03131427) since October 2014. 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 for the study from all patients.

The inclusion criteria were based on the American Association for the Study of Liver Diseases 2011 practice guidelines on haemochromatosis [6, 11]: (1) elevated ferritin (> 300 ng/mL in men and postmenopausal women or > 200 ng/mL in premenopausal women) and/or transferrin saturation (TS) ≥ 45%; (2) iron overload in the liver and/or spleen on magnetic resonance imaging (MRI) of the liver or liver histology.

The exclusion criteria: (1) alcoholic liver disease, chronic hepatitis B or C, or other chronic liver disease; (2) iron-overloading anemia; (3) parenteral iron overload.

**Clinical and laboratory profiles**

The following information were included in the studies: Sex; Age at diagnosis; laboratory data: serum ferritin (SF, a surrogate marker of storage iron), transferrin saturation (TS, the ratio of iron on transferrin); liver chemistry including ALT, AST, GGT, TBIL and ALB; clinical features at presentation: (a) liver fibrosis or cirrhosis, (b) skin pigmentation, (c) arthritis or arthropy, (d) cardiac involvement (including cardiomyopathy), (e) diabetes or hyperglycemia, (f) hypogonadism.

**Screening for gene variants**

Genomic DNA was extracted from whole blood using a Genomic DNA Purification Kit (Qiagen, Valencia, CA). All exons of known HH related genes (HFE, HJV, HAMP, TFR2 and SLC40A1) and exons with mutations in the novel iron homeostasis-related genes (DENND3 p. L708V and SUGP2 p.R639Q), were PCR-amplified with their associated boundary regions using primers described in our previous studies [10].

PCR amplification was performed in an ABI Veriti 96 PCR cycler (Applied Biosystems, MA, USA). PCR products were sequenced in forward and reverse orientations using an automated ABI 3730 DNA sequencer (Applied Biosystems). Three predictors, Polyphen-2 (http://genetics.bwh.harvard.edu), SIFT (http://sift.jcvi.org/) and Mutation Taster (http://www.mutationtaster.org/), were used to predict the functional consequence of the identified variants.

We defined pathogenic or likely pathogenic variants as those variants meeting one of the following criteria:

**Conclusions:** This is the first comprehensive analysis about the gene variant spectrum and phenotypic aspects of non-HFE HH in China. The results will be useful to the identification, diagnosis and management of HH in China.

**Keywords:** Hereditary haemochromatosis (HH), Non-HFE, Genotype, Genotype–phenotype
[12–14]: (1) the variants had previously been reported in the literature; (2) the variants were present in the HGMD, dbSNP, and ClinVar databases; (3) functional effect predictors predicted to be “damaging” by at least two of the three prediction tools were considered to be pathogenic variants; (4) the terms were used by standards and guidelines for the interpretation of sequence variants.

Statistical analysis
We used SPSS software V.26.0 to conduct all statistical comparisons. Continuous variables were presented as the mean ± standard deviation and compared using one-way ANOVA and LSD-t test, while continuous non-parametric variables were presented as median ± interquartile range and compared using Kruskal–Wallis ANOVA test. Discontinuous variables were compared using chi-square test and Fisher’s exact test. p Values of less than 0.05 was considered to be statistically significant.

Results
Clinical profiles of the enrolled patients
Thirty-two patients with primary iron overload from the CR-GMLD were recruited to screen for genetic variants in known HH-related genes and novel iron homeostasis-related genes. All the probands with primary iron overload were validated by liver biopsy and/or MRI examinations. Demographic characteristics of patients with non-HFE HH are shown in Table 1.

Gene variants distribution
We found that genetic variants forms of Chinese patients with primary iron overload are mainly non-HFE-related combined heterozygous variants. 1 case (3.13%) carried combined heterozygous HFE p.C282Y/71X pathogenic variants. 8 cases (25%) carried HJV pathogenic variants, among which 4 cases carried homozygous pathogenic variants in HJV gene, including p. Q6H, p. F103L, p. Q312X and p. C321X. 7 (21.88%) cases with SLC40A1 pathogenic variants, 8 (25%) cases with SUGP2 likely pathogenic variants, 5 (15.63%) cases with DENND3 likely pathogenic variants. None of damaging or probably damaging variants for HAMP and TFR2 was identified in any cases. Representative predicted pathogenic variants and allele frequency of HH included in this study are shown in Table 2. Representative pedigree analysis of four families are shown in Fig. 1.

Among these 31 non-HFE HH cases, 4 cases harbored homozygous variants, 2 cases harbored homozygous + heterozygous variants, 19 cases harbored heterozygous or combined heterozygous variants, and 6 cases harbored no damaging variants. Genetic characteristics of patients with HH are shown in Table 3.

| Table 1 | Demographic characteristics of patients in in different groups with HH |
|---------|-------------------------|-------------------------|-------------------------|-------------------------|
| Characteristic | Total (32) | HJV (n = 8) | SLC40A1 (n = 6) | SUGP2 or DENND3 (n = 10) |
| Male, n (%) | 23(71.9) | 6 (75) | 4 (66.7) | 8 (80) |
| Age, y | 45.07 ± 15.94 (18–79) | 30.13 ± 12.12 | 56.33 ± 6.83 a | 48.30 ± 16.99 a |
| SF, ng/ml | 2631.0 (1115.95, 6371.25) | 6153(3246.5,6922.5) | 5917.6 (2061.8,9333.8) | 972 ab (670.3,2467.1) |
| TS, % | 92.30 (81.30,96.10) | 94.85 (92.25,97.5) | 92.25 (60.43,95.75) | 85.0(58.5,96.7) |
| ALT, U/L | 72.0 (30.0,104.0) | 99.5(67.3,12) | 78.5(39.5,141.3) | 31(22.5,44.5) |
| AST, U/L | 62.8 (34.6,103.0) | 92(64.4,121.0) | 60(37.6,111.8) | 33.4(22.9,48.5) |
| GGT, U/L | 42(27.00,72.0) | 48(34,3,75) | 38(19,8,278.3) | 29.9(26.5,58.5) |
| TBIL, μmol/L | 37.88 ± 43.86 | 17.56 ± 8.85 | 18.79 ± 10.72 | 64.14 ± 62.59 a |
| ALB, g/L | 40.30 ± 8.33 | 43.98 ± 10.12 | 38.88 ± 3.87 | 39.14 ± 9.42 |
| Cirrhosis | 11 | 0 | 5 | 4 |
| Skin pigmentation | 13 | 4 | 4 | 2 |
| Arthropathy | 2 | 1 | 1 | 0 |
| Cardiac involvement | 5 | 3 | 0 | 0 |
| Diabetes | 11 | 3 | 5 | 1 |
| Hypogonadism | 9 | 5 | 3 | 1 |

HH without P or LP variants* = HH without pathogenic or likely pathogenic variants, SF = serum ferritin, TS = transferrin saturation, ALT = alanine transaminase, AST = aspartate aminotransferase, GGT = γ-glutamyltransferase, TBIL = total bilirubin, ALB = albumin; Statistically significant differences are denoted as (a) compared to HJV. Statistically significant differences are denoted as (b) compared to SLC40A1
Table 2  Representative predicted pathogenic variants and allele frequency of HH included in this study

| Gene | Location | Nucleotide change | Amino acid alteration | Mutation type | Frequency (this study) | Frequency (Asia/World) | Prediction of Pathogenicity (score) | Polyphen-2 | SIFT | Mutation Taster |
|------|----------|-------------------|----------------------|---------------|------------------------|------------------------|-----------------------------------|------------|------|-----------------|
| HJV  | Exon 4   | c.963C>A          | p.C321X              | Nonsense      | 7/32                   | 5.798e−05/4.062e−06   | NA                                | NA         | NA   | Disease causing (1) |
| HJV  | Exon 4   | c.842T>C          | p.I281T              | Missense      | 2/32                   | 5.798e−05/8.121e−06   | Probably damaging (1.0)           | NA         | NA   | Disease causing (0.999) |
| HJV  | Exon 4   | c.934C>T          | p.Q312X              | Nonsense      | 1/32                   | NA                    | Probably damaging (0.999)          | NA         | NA   | Disease causing (1) |
| HJV  | Exon 3   | c.311A>G          | p.H104R              | Missense      | 1/32                   | NA                    | Probably damaging (0.997)          | NA         | NA   | Disease causing (1) |
| HJV  | Exon 4   | c.820G>A          | p.V274M              | Missense      | 1/32                   | 0.0012/8.527e−05      | Probably damaging (0.986)          | NA         | NA   | Disease causing (1) |
| HJV  | Exon 3   | c.309C>G          | p.F103L              | Missense      | 1/32                   | NA                    | Probably damaging (0.649)          | NA         | NA   | Disease causing (1) |
| SLC40A1 | Exon 5 | c.430A>G          | p.N144D              | Missense      | 1/32                   | NA                    | Probably damaging (1.0)           | NA         | NA   | Disease causing (1) |
| SLC40A1 | Exon 7 | c.997T>C          | p.Y333H              | Missense      | 3/32                   | NA                    | Probably damaging (1.0)           | NA         | NA   | Disease causing (1) |
| SLC40A1 | Exon 8  | c.1531G>A         | p.V511I              | Missense      | 1/32                   | NA                    | Probably damaging (0.984)         | NA         | NA   | Disease causing (1) |
| SLC40A1 | Exon 5 | c.485_487delTTG   | p.v162del            | Deletion      | 1/32                   | NA                    | –                                 | –          | –    | –               |
| SLC40A1 | Intron | MS3+10delIGTT    |                     | Splicing      | –                      | NA                    | –                                 | –          | –    | –               |
| SUGP2 | Exon 5   | c.1916G>A         | p.R639Q              | Missense      | 8/32                   | 0.0388/0.0029         | Probably damaging (0.992)         | NA         | NA   | Disease causing (1) |
| DBNND3 | Exon 14 | c.2122C>G         | p.L708V              | Missense      | 5/32                   | 0.0333/0.0029         | Probably damaging (0.982)         | NA         | NA   | Disease causing (1) |
| HFE   | Exon 4   | c.845G>A          | p.C282Y              | Missense      | 1/32                   | 0.0001/0.0332         | Probably damaging (1.0)           | NA         | NA   | Disease causing (1) |
| HFE   | Exon 2   | c.211C>T          | p.R71X               | Nonsense      | 1/32                   | 0.0002/1.624e−05      | NA                                | NA         | NA   | Disease causing (1) |
Grouping of non-\textit{HFE} HH

Patients with non-\textit{HFE} related HH were divided into four groups in the study, \textit{HJV} HH (Type 2A HH), \textit{SLC40A1} HH (Type 4B HH), \textit{SUGP2} or \textit{DENND3} variants HH and No pathogenic or likely pathogenic variants HH groups, based on the pathogenic variants identified in these cases. Demographic and laboratory characteristics of the four groups of HH cases are shown in Table 1.

Among the 31 cases with non-\textit{HFE} related HH, 2 cases (the first is \textit{SLC40A1} p. Y333H + \textit{SLUGP2} p. R639Q, the second is \textit{SLC40A1} IVS3+10delGTT + \textit{SLUGP2} p. R639Q(homo)) carried two different pathogenic or likely pathogenic variants. The first was grouped into the \textit{SLC40A1} HH due to the definite pathogenicity of \textit{SLC40A1} p. Y333H. Previous functional studies showed that the \textit{SLC40A1} p. Y333H variant was associated with gain-of-function of ferroportin and caused iron overload and organ damage [15]. The second carried two likely pathogenic variants. This patient was a 48-year-old female with SF 7078 ng/ml and TS 99.7%. Liver biopsy suggested that iron deposition was predominant in hepatocytes, MRI suggested iron overload in liver and spleen, and gene test suggested \textit{SLC40A1} IVS3+10delGTT + \textit{SLUGP2} p. R639Q(homo) combined likely pathogenic variants. We excluded this patient due to difficulty in grouping. Therefore, we finally analyzed the remaining 30 cases.

Genotype and phenotype associations in different types of non-\textit{HFE} HH

\textit{HJV} HH (Type 2A HH)

There were more males than females in \textit{HJV} HH, the ratio of males and females was 3:1. Mean age at diagnosis of this group of patients was the lowest (30 years) in the four types. Totally, 62.5% of the patients had hypogonadism, half of them developed skin pigmentation, 37.5% had both cardiac involvement and diabetes. Only one case developed arthropathy. None of patients in \textit{HJV} HH developed cirrhosis.

ALT and AST (median 99.5 and 92 U/L) levels increased in this group. GGT (median 48 U/L), TBIL (mean 17.6 μmol/L) and ALB (mean 44.0 g/L) levels were normal. The median SF (6153 ng/ml) and TS (median 95%) levels were highest in four types.

\textit{SLC40A1} HH (Type 4B HH)

There were more males than females in \textit{SLC40A1} HH, the ratio of males and females was 2:1. Mean age at diagnosis
Table 3  Genetic characteristics of 32 patients in HH cases

| NO | Age  | Gender | SF (ng/ml) | TS (%) | Iron deposition on liver biopsy | Iron overload on MRI | Known HH-related genes | Iron homeostasis-related genes |
|----|------|--------|------------|--------|---------------------------------|---------------------|------------------------|-----------------------------|
| 1  | 26   | M      | 7004       | 92.0   | Predominant in hepatocytes      | Liver               | HJV p.Q6H/C321X/I281T  | –                           |
| 2  | 28   | M      | 6269       | 95.4   | Predominant in hepatocytes      | Liver, pancreas     | HJV p.Q6H/C321X/I281T  | –                           |
| 3  | 22   | F      | 2995       | 89.1   | Predominant in hepatocytes      | Liver, pancreas     | HJV p.Q312X (homo)     | –                           |
| 4  | 18   | M      | 6678       | 100    | Predominant in hepatocytes      | ND                  | HJV p.Q6H/C321X/H104R  | –                           |
| 5  | 57   | M      | 4001       | 93.0   | Predominant in hepatocytes      | Liver               | HJV p.Q6H/C321X/V274M  | –                           |
| 6  | 36   | F      | 2000       | 96.0   | Predominant in hepatocytes      | Liver               | HJV p.F103L (homo)     | –                           |
| 7  | 30   | M      | 11,555     | 98.0   | ND                              | Liver, spleen, pancreas | HJV p.Q6H (homo) / C321X (homo) | – |
| 8  | 24   | M      | 6037       | 94.3   | ND                              | Liver, pancreas     | HJV p.Q6H (homo) / C321X (homo) | – |
| 9  | 57   | F      | 5886.1     | 71.1   | Hepatocytes and reticuloendothelial cells | Liver, spleen, pancreas | SLC40A1 p. N144D     | –                           |
| 10 | 66   | F      | 1446.2     | 92.7   | ND                              | Liver, spleen, pancreas | SLC40A1 p. Y333H     | SUGP2 p. R639Q              |
| 11 | 48   | M      | 2267        | 91.8   | ND                              | Liver, spleen, pancreas | SLC40A1 p. V511I     | –                           |
| 12 | 58   | M      | 5949        | 28.4   | Predominant in hepatocytes      | Liver, spleen        | SLC40A1 p. v162del    | –                           |
| 13 | 49   | M      | 7445        | 97.0   | Predominant in hepatocytes      | Liver, spleen        | SLC40A1 p. Y333H     | –                           |
| 14 | 60   | M      | 15,000      | 94.0   | Predominant in hepatocytes      | Liver, spleen, pancreas | SLC40A1 p. Y333H     | –                           |
| 15 | 79   | M      | 493.8       | 97.1   | ND                              | Liver, pancreas     | SUGP2 p. R639Q       | –                           |
| 16 | 63   | M      | 3868        | 97.7   | ND                              | Liver, spleen        | SUGP2 p. R639Q       | –                           |
| 17 | 67   | M      | 1102        | 92.3   | Predominant in hepatocytes      | Liver, spleen        | DENND3 p. L708V(homo)| SUGP2 p. R639Q              |
| 18 | 28   | M      | 738         | 46.4   | ND                              | –                    | DENND3 p. L708V     | –                           |
| 19 | 38   | F      | 843         | 96.2   | Predominant in hepatocytes      | ND                  | –                      | –                           |
| 20 | 53   | F      | 1402        | 49.0   | Predominant in hepatocytes      | –                    | SUGP2 p. R639Q       | –                           |
| 21 | 46   | M      | 2000        | 85.0   | ND                              | –                    | SUGP2 p. R639Q       | –                           |
| 22 | 45   | M      | 685         | –      | Predominant in hepatocytes      | ND                  | –                      | –                           |
| 23 | 33   | M      | 626         | 68.0   | Hepatocytes and reticuloendothelial cells | –                    | –                      | SUGP2 p. R639Q              |
| 24 | 31   | M      | 6000        | 81.0   | ND                              | –                    | –                      | –                           |
| 25 | 66   | F      | 12,703      | 91.7   | Predominant in hepatocytes      | –                    | DENND3 p. L708V     | –                           |
| 26 | 50   | F      | 9272        | 81.6   | Predominant in hepatocytes      | –                    | –                      | –                           |
| 27 | 53   | M      | 1121        | 37.6   | Predominant in reticuloendothelial cells | –                    | –                      | –                           |
| 28 | 53   | M      | 773         | 96.8   | Predominant in hepatocytes      | –                    | –                      | –                           |
| 29 | 31   | M      | 1220        | 92.4   | ND                              | –                    | –                      | –                           |
| 30 | 37   | M      | 1316        | 85.0   | ND                              | –                    | –                      | –                           |
| 31 | 48   | F      | 7078        | 99.7   | Predominant in hepatocytes      | –                    | SLC40A1 IVS3 + 10delGTT | SUGP2 p. R639Q(homo)       |
| 32 | 28   | M      | 2153        | 91.7   | Predominant in hepatocytes      | –                    | HFE p.C282Y/R71X     | –                           |
of this group of patients was the highest (56 years) in the four types. Totally, 83.3% of the patients had both cirrhosis and diabetes, 66.6% had skin pigmentation, half of them had hypogonadism. Only one case had arthropathy. None of patients in SLC40A1 HH developed cardiac diseases.

ALT and AST (median 78.5 and 60.4 U/L) levels increased in this group. GGT (median 18.9 μmol/L), TBIL (mean 18.9 μmol/L), and ALB (mean 38.9 g/L) levels were normal. The median SF level was 5918 ng/ml. The median TS was 92%.

SUGP2 or DENND3 variants HH
There were more males than females in SUGP2 or DENND3 HH, the ratio of males and females was 4:1. Mean age at diagnosis of this group of patients was 48 years. Totally, 40% of the patients had cirrhosis, 20% had skin pigmentation, 10% had both diabetes and hypogonadism. None of patients in SUGP2 or DENND3 HH developed arthropathy and cardiac diseases.

ALT (median 31 U/L), AST (median 33.4 U/L), GGT (median 29.9 U/L), and ALB (mean 39.1 g/L) levels were normal in this group. TBIL (mean 64.1 μmol/L) levels were higher than other groups. The median SF level was 972 ng/ml. The median TS level was 85%.

HH without pathogenic or likely pathogenic variants
There were more males than females in HH without pathogenic or likely pathogenic variants, the ratio of males and females was 2:1. Mean age at diagnosis of this group of patients was 48 years. Totally, 50% of the patients had skin pigmentation, 33.3% had cirrhosis, 33.3% had both cardiac diseases and diabetes. None of patients developed arthropathy and hypogonadism.

ALT and AST (median 72 and 72 U/L), TBIL (mean 51.3 μmol/L) levels increased in this group. GGT (median 56 U/L) and ALB (mean 38.0 g/L) levels were normal. The median SF level was 1267 ng/ml. The median TS level was 88%.

There were more males than females in all groups (Fig. 2A). The age at diagnosis was statistically different between HJV HH and SLC40A1 HH groups (p = 0.001), between HJV HH and SUGP2 or DENND3 HH groups (p = 0.008), between HJV HH and HH without pathogenic or likely pathogenic variants groups (p = 0.018), while the comparison between other groups was not statistically significant (Fig. 2B). The incidence of cirrhosis (p = 0.011), cardiac involvement (p = 0.042), diabetes (p = 0.035) and hypogonadism (p = 0.020) was statistically significant in the four groups. However, due to the limited sample size, the pairwise comparison showed no significant difference (Fig. 3). HH without P or LP

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**Table 3 (continued)**

| HH = Hereditary hemochromatosis, ND = not done |

Fig. 2 Gender and age at diagnosis of patients with non-HFE-related HH. A Gender and B Age at diagnosis are shown for patients with HJV HH, SLC40A1 HH, SUGP2 or DENND3 HH and HH without pathogenic or likely pathogenic variants. Graphs A show the ratios of males and females in the four groups. Variables were compared using chi-square test and Fisher’s exact test. There are no statistical differences between the groups. Graphs B show percentage of patients at different age stages (< 30Y, 30-50Y, > 50Y). Variables were compared using one-way ANOVA and LSD-t test. Statistically significant differences are denoted as (a) compared to HJV.
variants* = HH without pathogenic or likely pathogenic variants.

TBIL levels were significantly higher in SUGP2 or DENND3 HH when compared to HJV HH groups ($p = 0.032$). There was no significant difference about other liver function indices in the four groups. SF levels increased greatly in all the four groups. There were statistical differences between HJV HH and SUGP2 or DENND3 HH groups ($p = 0.002$), between SLC40A1 HH and SUGP2 or DENND3 HH groups ($p = 0.01$), while the comparison between other groups was not statistically significant. TS increased greatly in all HH groups, with a median TS of 92% in all cases.

**Discussion**

The present study demonstrated that the pathogenic gene of HH in China was mainly non-HFE genes, which is different from that in Caucasians. We only found one case carried combined heterozygous HFE p.C282Y/71X pathogenic variant in our study, which has been reported in our previous study [16]. The remaining 31 cases are non-HFE-related HH, especially in HJV and SLC40A1 genes. This suggests that type 2A HH and type 4 HH are major forms of HH in Chinese populations. Also, we analyzed the correlation of the genotype and clinical phenotype of these HH patients.

In the present study, cases with HJV-related HH had an earlier age at diagnosis and more severe iron load. In contrast, SLC40A1 HH, SUGP2 or DENND3 HH and HH without pathogenic or likely pathogenic variants occur relatively late, rarely before the age of 30. Furthermore, most of the patients with HJV HH had heart failure as the first symptom, whereas there was no cirrhosis in HJV HH. A meta-analysis showed that cardiomyopathy is generally seen in individuals with much greater degrees of iron overload [17]. This is in line with the previous report that a much earlier and more serious deposition of iron in the heart in HJV HH [18]. However, the exact mechanism for the severer heart disease than the liver disease in HJV HH is still not clear.
We also found cases with SLC40A1 HH had higher prevalence of cirrhosis and diabetes in this studies, which may be related to the severe iron overload and the late onset age of SLC40A1 HH. Firstly, higher ferritin levels are independently associated with prevalent diabetes [19]. Some studies showed that increased ferritin was associated with increased risk of type 2 diabetes after adjustment for conventional risk factors for diabetes [20]. This relationship between iron and diabetes was also found in gestational diabetes and prediabetes [21, 22]. Secondly, age is known to be a risk factor for diabetes. The older age may increase the more chances of developing cirrhosis and diabetes than HJV HH. In addition, severe iron overload in pancreas was observed in some patients, which may be associated with the onset of diabetes.

In our previous study, function study showed that silencing S LigP2 expression downregulated the level of HAMP expression, and a decrease in the level of p-SMAD1/5 and TFR2 was observed in the Huh-7 cell line transfected with the DENND3 and DENND3 p. L708V constructs [10]. In the present study, cases with S LigP2 or DENND3 HH group showed lower involvement of skin pigmentation, arthropathy, cardiac diseases, diabetes and hypogonadism, when compared to HJV HH group; and lower prevalence of cirrhosis when compared to SLC40A1 HH group. This may be due to the lower accumulation of iron in S LigP2 or DENND3 HH group than HJV HH group and SLC40A1 HH group. Therefore, we may infer that the pathogenicity of S LigP2 or DENND3 gene variants is weaker than those in HJV or SLC40A1 gene.

It is worth noting that HH without pathogenic or likely pathogenic variations were identified in 6 cases, suggesting that pathogenic variants may exist in other HH-related genes. The second-generation sequencing for those unexplained HH cases would be justified.

Overall, our data showed that there were more men than women among these patients with different non-HFE genotypes. Relevant studies have found that the onset age of female patients is usually later than that of male patients [23]. The prevalence of high serum ferritin levels is higher in males than in females. We think the appearance of symptoms may also be associated to other non-genetic factors, such as alcohol and high-fat diet, and it may explain the protective effect against hemochromatosis by the fact that menstruation delay iron accumulation in women [23]. We found that in the HJV HH, SLC40A1 HH and HH without pathogenic or likely pathogenic variants groups, about half of cases had skin pigmentation, whereas arthropathy occurred in only two cases. This is consistent with the previous reports that skin pigmentation is more prevalent and arthropathy is rare in non-HFE HH compared to HFE HH [18, 24].

Studies have shown that there is significant positive correlations of SF with TS, ALT and AST [25]. The iron overload of type 2A and type 4 HH was higher than that of the other two groups. From the liver function tests in this study, ALT and AST increased in type 2A and type 4 HH. The rising trend of ALT/AST and SF in this study was consistent with the results of Barton J C [25].

Obviously, this study had some limitations. Firstly, the relatively small number of patients included preclude the conclusive correlation of genotype and phenotype. Secondly, we mainly focused on pathogenic or likely pathogenic variants and did not include the numerous combined heterozygous variants which might also confer various degree of pathogenicity. Thirdly, we did not analyze the histopathological characteristics, due to lack of liver biopsy in some patients.

Conclusions
In conclusion, this study suggested variants in non-HFE genes were the main pathogenic genes in Chinese HH patients. Cases with HJV-related HH had an earlier age at diagnosis and the more severe iron load, whereas more cases with SLC40A1 HH had cirrhosis and diabetes. S LigP2 and DENND3 were likely pathogenic variants for HH in China.

Abbreviations
HH: Hereditary hemochromatosis; HFE: Haemochromatosis; HJV: Hemojuvelin; HAMP: Hepcidin; TFR2: Transferrin receptor 2; SLC40A1: Ferroportin; DENND3: DENN domain-containing protein 3; S LigP2: SURP and G patch domain containing 2; MRI: Magnetic resonance imaging; SF: Serum ferritin; TS: Transferrin saturation; ALT: Alanine transaminase; AST: Aspartate aminotransferase; GGT: γ-Glutamyltransferase; TBIL: Total bilirubin; ALB: Albumin; PCR: Polymerase chain reaction.

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

Authors’ contributions
LW and WZ contributed equally to this work. LW, WZ, XO and JH participated in the design of the study. LW, WZ, YL, DJ, ZB and AX performed the targeted Sanger sequencing, sequencing data analysis and prediction of variants. LW, WZ, ZW, LW, SL, XW, XZ, QW, YW and HY performed recruitment of patients and collected clinical data. LW and ML did the statistical analysis. LW drafted the manuscript; LW helped to draft the manuscript; LW, WZ, JH, XO and JJ revised it critically for intellectual content. All authors read and approved the final manuscript.

Funding
This work was supported by grants from the National Key Technologies R&D Program of China (No. 2015BAI13B09), the National Natural Science Foundation of China (No. 82070598), the Youth Programme of Beijing Hospitals Authority (No. QML20190105), and the Digestive Medical Coordinated Development Center of Beijing Hospitals Authority (No. XXZ0502).
Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

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

Consent for publication
All authors agreed on the manuscript.

Competing interests
The authors declare that they have no competing interests.

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Received: 20 March 2021   Accepted: 20 September 2021
Published online: 28 September 2021

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