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Biochemical and Bioinformatic Characterization of Patients with a Sodium Taurocholate Cotransporting Polypeptide Mutation

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Abstract

Background: *SLC10A1* codes for the sodium taurocholate cotransporting polypeptide (NTCP). The *SLC10A1*^{S267F} mutation is associated with loss of function of bile acid (BA) uptake and defined as a new type of hypercholanemia. This kind of hypercholanemia is characterized by high levels of serum BA. However, limited studies have been conducted on this topic.

Objectives: This study aimed to describe the biochemical and bioinformatic characterization of patients with an *SLC10A1*^{S267F} mutation, as well as to dissect pathogenesis in hypercholanemia.

Methods: In this study, a total of 12 individuals (including 5 homozygous, 3 heterozygous, and 4 wild-type individuals) were recruited. Whole-genome sequencing (WGS) and Sanger sequencing were used to confirm the genotype. Tests of liver function, renal function, and serum lipid level, in addition to routine blood tests, were performed to evaluate the clinical consequences of patients with an *SLC10A1*^{S267F} mutation. The ClinVar website and protein prediction tools were used to analyze other cholesterol and BAs related gene mutations in *SLC10A1*^{S267F} patients, as well as to evaluate their possible effects on serum BA levels of patients.

Results: All *SLC10A1*^{S267F} homozygous patients displayed high levels of BAs. Liver and renal functions were generally normal. According to previous reports, homozygous patients are prone to vitamin D deficiency and deviated blood lipids. However, all homozygous individuals had normal levels of blood lipids, thyroid hormones, and vitamin D (25(OH)D). Moreover, except for the *SLC10A1*^{S267F} mutation, according to the WGS results, multiple gene mutations were found in 5 homozygous and might affect the level of BAs, but the *SLC10A1*^{S267F} mutation still is the most important reason resulting in a high level of BAs.

Conclusions: This study provided a more detailed description of the *SLC10A1*^{S267F} mutation-induced hypercholanemia, delivering a new idea that there might be some mutations in *SLC10A1*^{S267F} homozygotes, probably influencing BA metabolism.

Keywords: *SLC10A1*, Mutations, Whole-Genome Sequencing, p.Ser267Phe, Hypercholanemia

1. Background

Bile is mainly composed of bile salts, phospholipids, and cholesterol. Bile salt is a kind of sodium salt of the conjugate of bile acid (BA) with either glycine or taurine. The conversion of cholesterol to BAs is closely related to lipid metabolism, fat solute vitamins, and hormones (1). BA levels differ in different liver diseases, and the level of BA in the liver reflects and influences liver function to a certain extent. However, the long-term clinical consequences of high serum BA levels remain unknown.

Hepatic uptake of BA is mainly mediated by sodium-dependent or independent transporters. The sodium taurocholate cotransporting polypeptide (NTCP; gene symbol *SLC10A1* in humans) is a major transporter of conjugated BA from the serum compartment into hepatocytes (2, 3). Besides, NTCP also transports additional substrates, such as thyroid hormones and drugs (4, 5).

Mutations in the *SLC10A1* gene, whose cytogenetic location is on chromosome 14 (14q24.1), may alter the expression and/or function of the NTCP protein, thus influencing bile salts transportation (6-10). The single nucleotide variant (p.Ser267Phe/c.800C>T) of NTCP/*SLC10A1* is associated with high levels of BA. The minor allele frequency (MAF) of *SLC10A1*^{S267F} in East Asia was reported to be between 8% and 12% (9, 11, 12). The *SLC10A1*^{S267F} variant exhibits a near-complete loss of function for bile salt uptake; yet, it possesses a fully normal transport function for the non-BA substrates such as estrone sulfate (8). This indicates that Ser267 may be placed in a protein region specific for BA transport. Therefore, any clinically impactful changes in the *SLC10A1*^{S267F} variant may affect liver detoxification or in-

crease susceptibility to hepatotoxicity in patients.

According to recent references in which only a total of 48 individuals, including 38 children and 10 adults (13-19), have been reported as *SLC10A1*^{S267F} homozygous patients, the mutation of *SLC10A1*^{S267F} is probably related to elevated serum BAs (a condition often referred to as hypercholanemia). However, the biochemical description of this mutation and whether it is the only reason influencing BAs in patients remain unclear. In this study, we identified 5 *SLC10A1*^{S267F} homozygous patients and investigated the clinical manifestations and long-term consequences. Here, we also analyze if there exists a combination of multiple gene mutations to influence serum BA levels in *SLC10A1*^{S267F} patients.

2. Methods

2.1. Human Subject

A total of 12 Han Chinese individuals were recruited at the First Affiliated Hospital of Army Medical University. We identified that 5 individuals with high serum levels of BAs were *SLC10A1*^{S267F} homozygous, while the other 7 individuals were heterozygous (n = 3) and wild-type (n = 4). We collected basic clinical information and peripheral blood samples from the individuals. Lab examinations, including liver and renal function tests, serum lipids level tests, and routine blood tests, were performed for each subject. This study was approved by the Ethics Committee of the First Affiliated Hospital of Army Medical University, PLA (People's Liberation Army of China) (code: KY2021116). All the study subjects or their legal guardians signed written informed consent.

2.2. Whole-Genome Sequencing

We performed a whole-genome sequencing (WGS) analysis of 12 individuals in this study. Using TruSeq Nano DNA LT Sample Preparation Kits (Illumina Inc, San Diego, CA, USA), the libraries were constructed. Briefly, using an S220 Focused-Ultrasonicator (Covaris, USA), the genomic DNA was sheared into fragments with a length of ~350 base pairs (bp). Using an Illumina sequencing platform, a HiSeq X Ten platform (Illumina Inc, San Diego, CA, USA), the final libraries were sequenced after polymerase chain reaction (PCR) amplification and purification, and 150-bp paired-end reads were generated. The raw sequence data were processed using the NGS-QC toolkit (20) for quality control of sequence data. The clean reads were aligned to the reference genome (GRCh38/hg19) using the Burrows-Wheeler Aligner (21) (BWA), and the average coverage is 29.34X. After alignment, Picard was employed to mark duplicate reads, and SAMtools (22) was used to transform the reads into compressed binary alignment map (BAM) files for sorting and indexing. The genome analysis toolkit (GATK) (23) were used to verify the accuracy of single nucleotide polymorphisms (SNP) and insertion and deletion (InDel). Finally, ANNOVAR (24) was used to perform annotation for the variants.

2.3. Gene Variation Screening

After completing the annotation and filtering steps, we focused on genes known to code for cholesterol and BA metabolism. Firstly, we used "bile acid" and "cholesterol" as keywords and selected all genes coding for human cholesterol metabolism, especially for BA metabolism, using the UniProt database and obtained a total of 560 genes. We further retrieved the SNP file from the WGS results and obtained 709 SNPs (except for *SLC10A1*^{S267F}) from the recruited subjects. Because homozygotes have greater phenotype and clinical importance, we decided to narrow down the number of SNPs by excluding the heterozygous mutation, meaning that those SNP genotypes should be homozygous in individuals 1 to 5. A total of 560 genes were filtered and reduced to 62 genes covering 76 missense variants. Secondly, to evaluate benign or deleterious of those variants, we searched for their clinical significance in ClinVar or from previous studies and classified them as benign or deleterious variants. Meanwhile, for mutations not previously reported, we used MAF < 0.05 in the public database of 1000 genomes project for the East Asian population and protein prediction tools, including sorting tolerant from intolerant (SIFT; dbNSFP version 3.3a) and polymorphism phenotyping version 2 (Polyphen 2 version 2.2), to forecast the underlying functional impacts. The protein function prediction of SIFT is based on the SIFT score calculated by sequence homology and the physical/chemical similarity between amino acids (deleterious means sift $\leq$ 0.05; tolerated means sift $>$ 0.05). On the other hand, we used the polyphen 2 HDIV score for protein function prediction, which is based on the HumanDiv database for complex diseases (probably damaging: score $\geq$ 0.957; possibly damaging: Score = 0.453 ~ 0.956; benign: score $\leq$ 0.452). Besides, we examined the pathogenesis of the variants reported in all related studies. There were 31 possibly damaging variations that might be related to hypercholanemia (Table 1).

2.4. Sanger Sequencing Validation

Sanger sequencing was used to confirm the *SLC10A1*^{S267F} mutation in all recruited individuals. PCR primers were designed and used to amplify the 309/419-bp fragment covering the *SLC10A1* gene (forward primer, 5'-GCTAGAAACTTGCTTGTTG-3'; reverse primer, 5'-CTCTGAGTGTATGTGGGGT-3' or forward primer, 5'-GAGTGCAGTTGATGGAAGT-3'; reverse primer, 5'-CTGAGTGTATGTGGGGTTT-3'). The *SLC10A1* gene information was obtained from the National Center for Biotechnology Information database.

Table 1. Variations in Bile Acids and Cholesterol Metabolism Genes, Detected by Whole - Genome Sequencing

Gene	rs	Variation		Clinical Significance	MAF	Functional Effects [a]	Genotype of Individual 1/2/3/4/5/6/7/8/9/10/11/12
ABCA12	rs7560008	c.T1375A	p.S459T	Benign	0.0016	Tolerated	HO/HO/HO/HO/HO/HO/HO/HO/HO/HO/HO/HO
ABCB11	rs2287622	c.T1331C	p.V444A	Progressive familial intrahepatic cholestasis	> 0.01	Tolerated	HO/HO/HO/HO/HO/HO/HO/HO/HO/HO/HE/HO
ABCC2	rs927344	c.A116T	p.Y39F	Dubin - Johnson syndrome	0.0054	Tolerated	HO/HO/HO/HO/HO/HO/HO/HO/HO/HO/HO/HO
ADGRG6	rs1262686	c.A3296G	p.Q1099R	Benign	0.0004	Deleterious	HO/HO/HO/HO/HO/HO/HO/HO/HO/HO/HO/HO
AKR1C4	rs4880718	c.A749G	p.Q250R	/	0.0002	Tolerated	HO/HO/HO/HO/HO/HO/HO/HO/HO/HO/HO/HO
APOB	rs679899	c.C1853T	p.A618V	Familial hypercholesterolemia, Familial hypobetalipoproteinemia	> 0.01	Deleterious	HO/HO/HO/HO/HO/HO/HO/HO/HO/HO/HO/HO
APOB	rs584542	c.A6937G	p.I2313V	/	> 0.01	Deleterious	HO/HO/HO/HO/HO/HO/HO/HO/HO/HO/HE/HO
APOL1	rs2239785	c.G394A	p.E132K	/	> 0.01	Deleterious	HO/HO/HO/HO/HO/HO/HO/HO/HO/HE/HO/HE
APOL1	rs136175	c.G630A	p.M210I	/	> 0.01	Deleterious	HO/HO/HO/HO/HO/HO/HO/HO/HO/HE/HO/HE
ATP11C	rs2491014	c.T342G	p.C114W	/	0.0079	Tolerated	HO/HO/HO/HO/HO/HO/HO/HO/HO/HO/HO/HO
ATP8B1	rs222581	c.G3454A	p.A1152T	/	0.0002	Deleterious	HO/HO/HO/HO/HO/HO/HO/HO/HO/HO/HO/HO
CD44	rs1467558	c.T689C	p.I230T	/	> 0.01	Deleterious	HO/HO/HO/HO/HO/HO/HO/HO/HO/HO/HO/HO
CTAGE5	rs7140561	c.T17C	p.V6A	/	> 0.01	Deleterious	HO/HO/HO/HO/HO/HO/HE/HE/WT/HE/HE/HE
CTAGE5	rs1950952	c.G991C	p.E331Q	/	> 0.01	Deleterious	HO/HO/HO/HO/HO/HO/HO/HO/HO/HO/HO/HO
CUBN	rs2796835	c.C8150G	p.S2717W	Benign	0.0000	Tolerated	HO/HO/HO/HO/HO/HO/HO/HO/HO/HO/HO/HO
CUBN	rs1276712	c.G6485A	p.C2162Y	Benign	0.0066	Tolerated	HO/HO/HO/HO/HO/HO/HO/HO/HO/HO/HO/HO
DBT	rs12021720	c.A1150G	p.S384G	Benign	> 0.01	Deleterious	HO/HO/HO/HO/HO/HO/HO/HO/HO/HO/HO/HO
EGF	rs4698803	c.A2633T	p.E878V	Benign	> 0.01	Deleterious	HO/HO/HO/HO/HO/HO/HO/HO/HO/HO/HO/HO
FCGBP	rs782538403	c.G3650A	p.R1217Q	/	0.0020	Tolerated	HO/HO/HO/HO/HO/HO/HO/HO/HO/HO/HO/HO
FCGBP	rs782416774	c.A3700G	p.T1234A	/	0.0048	Tolerated	HO/HO/HO/HO/HO/HO/HO/HO/HO/HO/HO/HO
FCGBP	rs782342257	c.G3707A	p.R1236Q	/	None	Deleterious	HO/HO/HO/HO/HO/HO/HO/HO/HO/HO/HO/HO
GC	rs9016	c.A1334G	p.H445R	/	0.0072	Deleterious	HO/HO/HO/HO/HO/HO/HO/HO/HO/HO/HO/HO
LIPC	rs6083	c.A644G	p.N215S	Hepatic lipase deficiency	> 0.01	Tolerated	HO/HO/HO/HO/HO/HO/HO/HO/HO/HO/HO/HO
LIPC	rs3829462	c.C1068A	p.F356L	Hepatic lipase deficiency	> 0.01	Tolerated	HO/HO/HO/HO/HO/HO/HO/HO/HE/HE/HO/HE/HO
PCSK9	rs562556	c.G1420A	p.V474I	Familial hypercholesterolemia, Familial hypobetalipoproteinemia	> 0.01	Tolerated	HO/HO/HO/HO/HO/HO/HO/HO/HO/HO/HO/HO
PEX16	rs10742772	c.G346A	p.V116I	Benign	0.0000	Tolerated	HO/HO/HO/HO/HO/HO/HO/HO/HO/HO/HO/HO
PROM2	rs12992066	c.A1523G	p.Q508R	/	> 0.01	Deleterious	HO/HO/HO/HO/HO/HO/HE/HO/HO/HO/HE/HE
SLC14A1	rs11877062	c.C10T	p.R4W	/	> 0.01	Deleterious	HO/HO/HO/HO/HO/HO/HO/HO/HO/HE/HE/HO
TF	rs2692696	c.A1342G	p.I448V	/	0.0068	Tolerated	HO/HO/HO/HO/HO/HO/HO/HO/HO/HO/HO/HO
TTC39B	rs1407977	c.A1051G	p.I351V	/	> 0.01	Deleterious	HO/HO/HO/HO/HO/HO/HO/HO/HO/HO/HO/HO
VPS33B	rs11073964	c.G1459A	p.G487S	Arthrogryposis with renal dysfunction and cholestasis syndrome	> 0.01	Deleterious	HO/HO/HO/HO/HO/HO/HO/HO/HO/HO/HO/HO

Abbreviations: MAF, minor allele frequency; HO, homozygous; HE, heterozygous; WT, wild - type.
[a] The functional effect was predicted by sorting tolerant from intolerant and polyphen.

2.5. Statistical Analysis

Data were analyzed using SPSS version 23 (SPSS Inc, Chicago, Ill, USA). Continuous variables with normal distribution were presented as mean ± SEM; non-normal variables were reported as median (interquartile range). A comparison of the clinical laboratory results among the 3 study groups (wild-type, heterozygote, and homozygote) was analyzed by either parametric or non-parametric tests (1-way analysis of variance [ANOVA] or Kruskal-Wallis omnibus test) as appropriate. P values less than 0.05 were considered statistically significant.

3. Results

3.1. Description of the Phenotype

We recruited a total of 12 subjects and divided them into 3 groups; homozygote, heterozygote, and wild-type (Figure 1; Table 2). Telephone follow-ups revealed good cognition competence and a normal social ability for both adults and children. Adults of homozygotes and heterozygotes had well-developed secondary sexual characteristics and were fertile. Consistent with previous studies (13-19), the level of serum BA was much higher in homozygotes than in wild-type individuals (P = 0.027), meaning that all 5 homozygotes had hypercholanemia, whereas heterozygotes and wild-type individuals had normal serum BA levels. Many factors could affect serum BA levels, needing to be fully excluded before diagnosing *SLC10A1*^{S267F} mutation-induced hypercholanemia. Thus, a comprehensive medical history was obtained from each homozygote to determine any other diseases that could lead to high levels of serum BA. However, we did not find any individuals with symptoms of cholestatic liver diseases/malabsorptive diseases or long-term medication history.

We compared liver and renal functions between the 3 groups (Table 3). The levels of biochemical markers of liver injury were normal, except for high alkaline phosphatase activity in children because of the accelerated growth. The globulin levels decreased but not significantly. Also, the A/G ratio increased in the homozygotes (P = 0.036) and heterozygotes (P = 0.039) but with no clinical phenotype. The level of total bilirubin was lower in homozygotes than in wild-type individuals (P = 0.014) and heterozygotes but still within the normal range; the decreased total bilirubin might influence iron absorption in the long term in homozygotes.

Apart from the liver and renal functions, we also determined the levels of blood lipids (including total cholesterol, triglyceride, high-density lipoprotein cholesterol [HDL-C], and low-density lipoprotein cholesterol [LDL-C]), thyroid hormones (T3, T4, FT3, FT4, TSH), and vitamin D (25(OH)D). Previous studies (13, 14, 16) have reported that patients with a homozygous *SLC10A1*^{S267F} mutation were prone to vitamin D deficiency and abnormal blood lipid levels. However, in our study, all homozygotes had normal blood lipids, thyroid hormones, and vitamin D levels (Table 3). Compared to heterozygotes and wild-type subjects, in homozygotes, some indices (such as T3, T4, FT3, and FT4) increased while TSH decreased; however, all parameters were within the reference range.

The clinical chemistry parameters are presented in Table 1. The neutrophil count in homozygotes decreased (P = 0.015), whereas the lymphocyte and monocyte counts were slightly elevated. Some biochemical markers, including platelet (PLT) and platelet distribution width (PDW) in homozygotes, significantly differed compared with the heterozygotes (P = 0.001) and wild-type subjects (P < 0.001), respectively–but with a small clinical difference (Table 3). Interestingly, in heterozygotes, the lymphocyte count and PLT significantly decreased (P = 0.046 and P = 0.09), and PDW increased (P < 0.01). Taken together, these findings suggest that individuals with or without an *SLC10A1*^{S267F} mutation are healthy. However, the small differences in results should be studied further.

3.2. Validation of the *SLC10A1*^{S267F} Mutation Using WGS and Sanger Sequencing

WGS and Sanger sequencing were performed to confirm the presence of the *SLC10A1*^{S267F} mutation in the subjects. Individuals 1 to 5 were homozygotes, individuals 2 to 7 were heterozygotes at the variation allele, and individuals 9 to 12 were wild-type (Figure 1B). The Sanger sequencing results were consistent with WGS results.

3.3. Relationship of the *SLC10A1*^{S267F} Mutation and Other Gene Mutations in Homozygotes

We noted that in some cases, *SLC10A1*^{S267F} homozygotes with a combination of other gene mutations or diseases showed different symptoms, such as jaundice and/or liver dysfunction, late fetal loss, or urticarial rashes (13, 15, 17, 25, 26). We confirmed that serum BA levels increased asymptotically in *SLC10A1*^{S267F} homozygotes. Approximately 90% of cholesterol would be converted into BA by a series of enzymes and reactions in the liver. Therefore, cholesterol metabolism-related gene mutations could affect BA metabolism and might influence serum BA levels. To confirm if other variants could influence serum BA levels in homozygotes, we performed further gene analysis on these individuals. A total of 62 genes (coding for cholesterol metabolism and/or BA metabolism) covering 76 SNP were selected. The ClinVar public database and MAF were used to evaluate those variants. We also used protein prediction tools to predict underlying functional impacts in metabolism and find out deleterious variants. After filtering, 31 possibly damaging SNP were identified (see Table 1). According to references and prediction tools, except

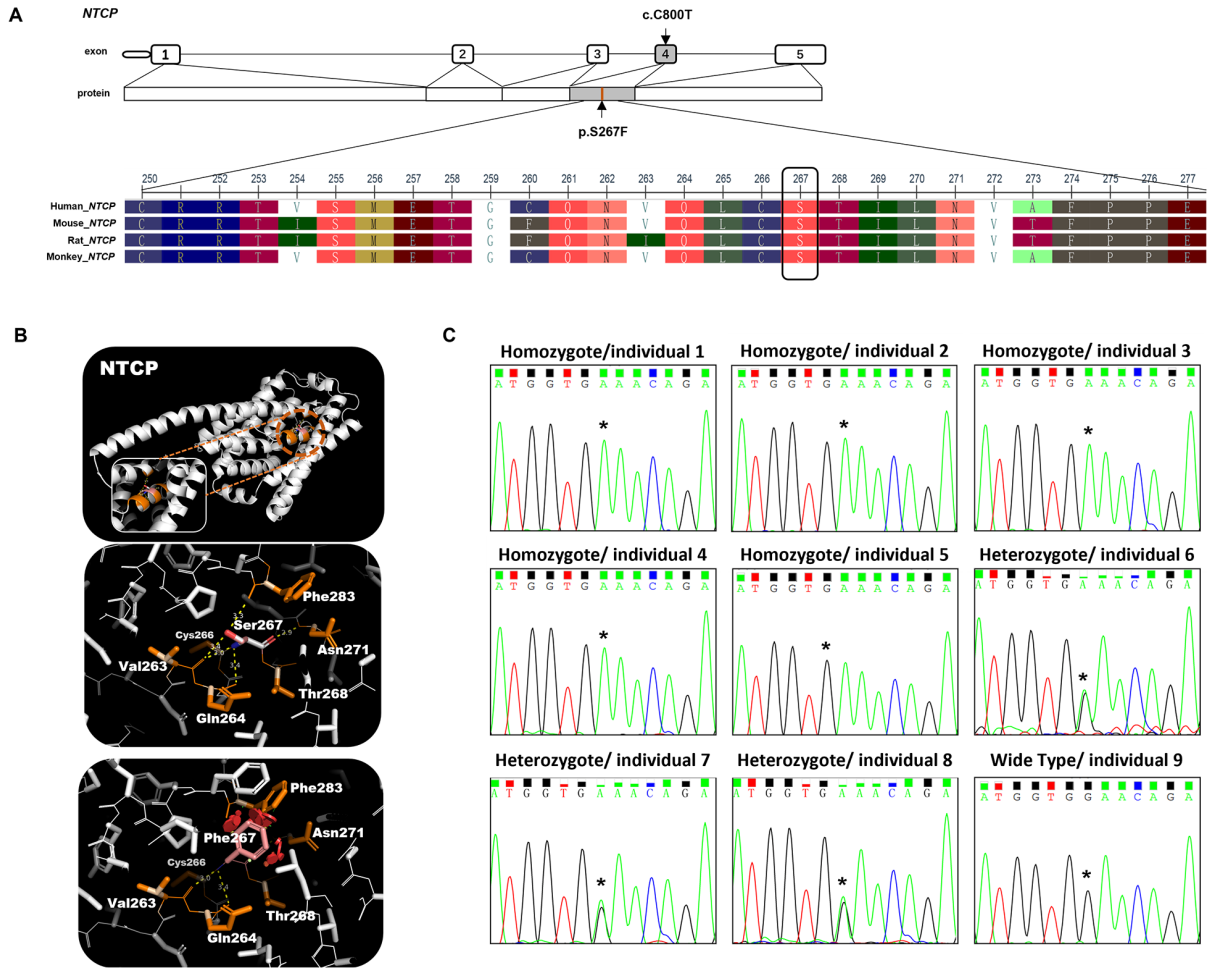

Figure 1. Bioinformatic analysis of the *SLC10A1* gene mutation. A, schematic view of NTCP exons and multiple sequence alignment information of NTCP; B, three - dimensional structures, prediction of NTCP wild - type and mutation; C, the Sanger sequencing results of the *SLC10A1* gene in 9 individuals. Individuals 1 to 5 were homozygous for the *SLC10A1* variants c.800c> T (p.Ser267Phe), individuals 6 to 8 were heterozygous for the *SLC10A1* variants c.800c> T (p.Ser267Phe), and individual 9 was wild - type for the *SLC10A1* variants c.800c> T (p.Ser267Phe). Individuals 10 to 12 were not included in this analysis due to the limited peripheral blood sample.

Table 2. Clinical Characteristics of Individuals

Variables	Homozygotes					Heterozygotes			Wild-Type			
Individual	1	2	3	4	5	6	7	8	9	10	11	12
Sex	Male	Male	Female	Female	Female	Female	Male	Female	Female	Male	Female	Male
Age (y)	36	2	11	7	5	36	33	31	9 months	43	44	17
TBA (umol/L)	33.1	7	34	35.2	47	1.9	4.9	287.7	2.8	3.6	1.7	9.1

Abbreviation: TBA, total bile acids.

for the *SLC10A1*^{S267F} mutation, there are 5 SNPs that have been thought to be the most possible factors in influencing serum BA levels in recruited homozygotes (*ABCB11*^{V444A}, *ABCC2*^{Y39F}, *APOB*^{A618V}, *PSCK9*^{V474I}, and *VPS33B*^{G487S}). None of the previous studies about *SLC10A1*^{S267F} have mentioned these SNPs, and *SLC10A1*^{S267F} in homozygotes was thought to be the most likely cause of high BA levels. Although we did not figure out the relationship between the 5 SNPs and hypercholesterolemia in this study, these variations will be studied in our future research.

4. Discussion

Non-synonymous variants of *SLC10A1*, including *c.668T>C(p.Ile223Thr)*, *c.800C>T(p.Ser267Phe)*, *c.836T>C(p.Ile279Thr)*, *c.940A>G(p.Lys314Glu)*, and *c.755G>A(p.Arg252His)*, have been reported previously. The p.Ser267Phe variant exhibits a loss of function for bile salt uptake, but it keeps a fully normal transport function for the non-BA substrate.

In this study, a biochemical assessment of recruited individuals was done to enrich the characterization of the *SLC10A1*^{S267F} mutation, which is more comprehensive compared with previous studies. Bioinformatics and clinical evidence in this study suggested that this variant was none-pathogenic, which is consistent with previously reported cases of NTCP deficiency. However, the neutrophil count in 2 children (individuals 3 and 8) was lower than normal, while their lymphocyte count was above normal. Two children with a homozygous *SLC10A1*^{S267F} mutation also presented decreased globulin, suggesting that researchers and doctors should focus on the immunologic function of homozygotes and their susceptibility to pathogens.

Bile and bile salts are vital for endogenous compounds and metabolic products, such as the digestion of dietary fat and fat-soluble vitamins (27-30). According to previous studies, fat-soluble compounds (such as vitamin D) deviate from the normal range in *SLC10A1*^{S267F} homozygotes. However, our patients displayed slight changes in vitamin D and serum lipid levels. Thus, the specific molecular role of *SLC10A1*^{S267F} in fat solubilization and absorption needs to be further elucidated.

Some studies have mentioned that patients with *SLC10A1*^{S267F} display worse clinical phenotype (such as liver dysfunction), always accompanied by other damage variants. Also, our previous results indicated that *Slc10a1*^{S267F} homozygous mice displayed normal levels of serum BA and did not cause hypercholanemia (26). Thus, to our knowledge, there are probably other variants in *SLC10A1*^{S267F} homozygotes; in this regard, 5 candidate SNPs are described below.

ABCB11 and *ABCC2* are genes coding for the bile salt export pump (BSEP) and multidrug resistance-associated protein 2 (MRP2), respectively. BSEP and MRP2 are bile salt transporters located at the canalicular membrane of hepatocytes (6). BSEP mainly participates in hepatic BA and lipid homeostasis. In most cases, mutations in BSEP are closely related to inherited progressive cholestatic liver disorders in infancy, known as progressive familial intrahepatic cholestasis (PFIC) (31, 32). Missense variant *ABCB11*^{V444A} is related not only to PFIC but also to a risk factor of intrahepatic cholestasis in pregnancy or drug-induced liver injury (33-35). Therefore, we speculate that 444Ala constitutes a susceptibility factor for the development of hypercholanemia under certain conditions, such as a combination mutation of *SLC10A1*^{S267F}. MRP2 can transport divalent bile salts. The *ABCC2*^{Y39F} mutation has found in *SLC10A1*^{S267F} homozygotes. Some mutations of single amino acid in *ABCC2* could confer MRP2 new transport capacity for bile salts, indicating that we should pay attention to this SNP and its underlying effect on serum BA levels.

Mutations of *APOB*^{A618V} and *PSCK9*^{V474I} were reported as related factors of familial hypercholesterolemia in the ClinVar database. Apolipoprotein B (*APOB*) is a major protein constituent of chylomicrons (apo B-48), LDL (apo B-100), and VLDL (apo B-100) (36). The *APOB*^{A618V} mutation is associated with higher LDL-C levels (odds ratio [OR] = 1.41) (37), meaning that cholesterol and BA levels in *SLC10A1*^{S267F} homozygotes could be influenced to some extent. The coexistence of *APOB*^{A618V} and *APOB*^{A618V} should be studied. The main function of *PCSK9* is the degradation of the LDL receptor. In some cases, *PCSK9* increases apoB100 production, reduces *APOB* degradation to increase circulating LDL levels and contributes to hypercholesterolemia and atherosclerosis (38). However, *PSCK9*^{V474I} seems to be a protection variant associated with lower LDL and cholesterol (39). This might be the reason why the serum lipids in *SLC10A1*^{S267F} homozygotes were normal in this study.

VPS33B is a class C vacuolar protein sorting protein (40). Approximately 75% of arthrogryposis, renal dysfunction, and cholestasis syndrome (ARC) patients harbor germline mutations in *VPS33B*. ARC is characterized by congenital joint contractures, renal tubular acidosis, and neonatal jaundice, leading to death in infancy. However, in milder cases, patients survive into adulthood and increase serum BA values but normal bilirubin levels (41). The *VPS33B*^{G487S} mutation in *SLC10A1*^{S267F} homozygotes is predicted as a non-specific risk factor in ARC. However, the relationship between *VPS33B*^{G487S} and *SLC10A1*^{S267F} mutation still needs to be studied.

From the above discussion, we suggest that although the *SLC10A1*^{S267F} mutation is the most important reason for high serum BA levels, we detected some mutations in homozygotes that might influence BA metabolism. Further studies on these variants could provide a deeper understanding of the etiology of hypercholesterolemia.

Although we provided a comprehensive clinical, biochemical explanation of the *SLC10A1*^{S267F} mutation and discussed how to explore the etiology of the *SLC10A1*^{S267F} mutation-related hypercholesterolemia, there are some limitations. Due to experimental conditions and equipment limitations, we only used medical history data to exclude cholestasis/ malabsorptive diseases or long-term medication history (such as anti-hyperlipidemic agents). Also, we did not evaluate the insulin resistance status of the subjects.

Apart from the function of transporting molecules, NTCP has other functions. Convincing human genetic ev-

idence supports the role of NTCP as a cellular receptor for hepatic B virus (HBV). Recently, several epidemiology studies have reported *SLC10A1*^{S267F} homozygotes with lower HBV entry and hepatocyte infection compared to wild-type individuals. *SLC10A1*^{S267F} homozygotes may escape from HBV infection and present decreased cholesterol levels as a consequence of impaired BA uptake (42). According to the marked higher allele frequency of p.S267F in Asia, this SNP might be a positive selection of Asian for its advantage in conferring resistance to chronic HBV. We aim to design more in vivo and in vitro experiments to study and analyze these variants of *SLC10A1* in the next phase of our investigation. We have already constructed *Slc10a1*^{S267F} mutation mice and prepared for further study to determine whether there are deleterious effects on the population and how *267Phe* influences HBV infection. To understand the etiology of hypercholanemia, a mouse model of a *Slc10a1*^{S267F} mutation combined with other related variants is also needed. The effect of *p.Ser267Phe* on other BA transport proteins should also be explored.

4.1. Conclusions

This study presents an in-depth characterization of NTCP *p.Ser267Phe* mutations in 5 homozygotes by clinical examination, genetic analysis, and functional prediction. The results supported that the *p.Ser267Phe* mutation has no deleterious clinical effects in both children and adults.

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Footnotes

Authors' Contribution: Study concept and design: JIN CHAI; clinical data collection: XUEQIAN ZHOU, LIANGJUN ZHANG, and XIAOXUN ZHANG; analysis and interpretation of data: XUAN LI and HONG YANG; statistical analysis and drafting of the manuscript: XUAN LI.

Conflict of Interests: No conflicts of interest exist in the submission of this manuscript, and the manuscript is approved by all authors for publication.

Data Reproducibility: The dataset presented in the study is available on request from the corresponding author during submission or after its publication. The data are not publicly available due to the Regulations on the Management of Human Genetic Resources in China.

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Table 3. Biochemical Parameters of the Study Cohort Grouped by Genotype [a]

Genotype (rs2296651)			Wild-Type (GG) (n = 4)	Heterozygous (GA) (n = 3)	Homozygous (AA) (n = 5)
Test	Ref.	Unit			
Liver/Renal Function					
ALT	0 - 42	(IU/L)	14.87 ± 2.76	21.00 ± 18.25	20.00 ± 11.77
AST	0 - 42	(IU/L)	17.70 (17.65, 23.35)	16.00 (15.50, 21.50)	30.00 (26.00, 42.00)
ALP	34 - 114	(IU/L)	192.33 ± 199.95	59.33 ± 32.32	190.20 ± 90.73
GGT	5 - 50	(IU/L)	13.67 ± 3.21	20.67 ± 9.07	20.67 ± 7.20
TBA	0 - 10	(umol/L)	2.80 (2.25, 3.20)	4.90 (3.40, 5.95)	35.20 (34.00, 47.00) [b]
TBIL	6 - 21	(umol/L)	12.70 ± 3.93	11.03 ± 1.37	6.82 ± 2.17 [b]
DBIL	0 - 6	(umol/L)	2.57 ± 0.67	4.50 ± 1.84	2.98 ± 0.89
TP	66 - 83	(g/L)	72.83 ± 0.90	72.30 ± 1.93	70.40 ± 5.69
Alb	38 - 51	(g/L)	46.60 (45.40, 47.20)	45.20 (45.0, 46.6)	49.00 (48.80, 49.00)
G	25 - 38	(g/L)	26.63 ± 2.72	24.00 ± 5.19	20.84 ± 4.09
A/G	1.2 - 2.5		1.76 ± 0.25	1.77 ± 0.17 [b]	2.45 ± 0.49 [b]
UA	155 - 428	(umol/L)	374.67 ± 124.64	286.33 ± 138.60	277.80 ± 87.42
Urea	1.7 - 8.3	(mmol/L)	3.97 ± 1.07	3.81 ± 0.63	3.12 ± 1.26
Cre	45 - 84	(umol/L)	61.40 (46.20, 61.6)	43.00 (42.50, 57.35)	36.00 (21.00, 38.00)
Blood Fat					
TCH	3.1 - 5.7	(mmol/L)	4.63 ± 0.30	4.00 ± 1.14	4.16 ± 0.90
TG	0.4 - 1.73	(mmol/L)	1.28 (1.02, 2.185)	0.76 (0.53, 1.56)	1.07 (0.78, 1.43)
HDL - C	0.9 - 2	(mmol/L)	1.49 ± 0.52	1.44 ± 0.39	1.52 ± 0.31
LDL - C	2.07 - 3.1	(mmol/L)	2.73 ± 0.28	2.36 ± 0.81	2.64 ± 0.91
Serum Thyroid Hormone					
T3	1.3 - 3.1	(nmol/L)	1.35 (1.30, 1.58)	1.70 (1.65, 1.76)	2.09 (1.86, 2.16)
T4	66 - 181	(nmol/L)	98.70 (94.41, 121.22)	91.43 (89.87, 94.35)	110.05 (104.74, 144.10)
FT3	3.1 - 6.8	(pmol/L)	2.97 ± 0.70	2.57 ± 0.09	3.28 ± 0.52
FT4	12 - 22	(pmol/L)	15.82 (15.69, 16.55)	16.49 (14.96, 17.09)	21.49 (18.22, 23.09)
TSH	0.27 - 4.20	(uIU/mL)	0.78 ± 0.33	1.01 ± 0.39	0.56 ± 0.19
Vitamin D					
Vitamin D (25(OH)D)	25 - 200	(pmol/L)	50.60 (47.18, 55.02)	45.06 (42.86, 47.55)	32.26 (30.60, 34.57)
Routine Blood Tests					
WBC	3.5 - 9.5	(10^9/L)	7.78 ± 1.00	5.69 ± 0.64	7.48 ± 2.56
NEU	1.8 - 6.3	(10^9/L)	4.47 ± 0.81	3.33 ± 0.71	2.62 ± 0.88 [b]
LYM	1.1 - 3.2	(10^9/L)	2.79 ± 0.30	1.79 ± 0.10 [b]	3.97 ± 2.74
MON	0.1 - 0.6	(10^9/L)	0.35 ± 0.16	0.39 ± 0.01	0.51 ± 0.21
BAS	0 - 0.06	(10^9/L)	0.00 (0.00.0.11)	0.01 (0.005, 0.01)	0.02 (0.01, 0.03)
EOS	0.02 - 0.52	(10^9/L)	0.13 (0.09.0.21)	0.20 (0.12, 0.23)	0.15 (0.05, 0.17)
RBC	4.3 - 5.8	(10^{12}/L)	4.88 ± 0.33	4.95 ± 0.50	4.79 ± 0.56
HGB	115 - 150	(g/L)	141.00 ± 9.64	141.00 ± 24.02	127.80 ± 17.58
MCV	82 - 100	(%)	86.57 ± 5.26	88.13 ± 12.63	86.04 ± 13.52
MCH	27 - 34	(pg)	28.93 ± 1.87	28.43 ± 4.87	26.92 ± 3.71

MCHC	316 - 354	(g/L)	333.00 (333.00, 335.00)	318.00 (313.00, 331.00)	311.00 (309.00, 317.00)
RDW - SD	37 - 54	(%)	41.20 ± 1.01	46.07 ± 3.56 [b]	43.58 ± 1.82
RDW - CV	11 - 16	(%)	13.00 (12.75, 13.50)	13.70 (13.25, 14.95)	14.10 (14.00, 14.40)
PLT	125 - 350	(10^9/L)	287.00 ± 51.47	195.67 ± 17.67 [b]	310.40 ± 25.15 [c]
MPV	9 - 13	(fl)	9.70 ± 0.26	9.47 ± 1.02	9.66 ± 0.79
PDW	9 - 17	(fl)	11.50 ± 0.10	16.13 ± 0.90 [b]	15.66 ± 0.52 [b]

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; TBA, total bile acid; TBIL, total bilirubin; DBIL, direct bilirubin; TP, total protein; Alb, albumin; G, Globulin; UA, uric acid; Cre, creatinine; TCH, total cholesterol; TG, triglyceride; HDL - C, high - density lipoprotein cholesterol; LDL - C, low - density lipoprotein cholesterol; T3, triiodothyronine; T4, thyroxine, FT3, free triiodothyronine; FT4, free thyroxine; TSH, thyroid - stimulating hormone; WBC, white blood cell count; NEU, neutrophil; LYM, lymphocyte; MON, monocytes; BAS, basophils; EOS, eosinophil; RBC, red blood cell count; HGB, hemoglobin; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; , red blood cell distribution width (RDW), RBC distribution width; SD, standard deviation; PLT, platelet; CV, coefficient of variation; MPV, mean platelet volume; PDW, platelet distribution width; GG, the wild - type for p.Ser267Phe in *SLC10A1*; GA, the heterozygote for p.Ser267Phe in *SLC10A1*; AA, the homozygote for p.Ser267Phe in *SLC10A1*.

[a] Values are presented as means ± SDs/SEMs or Mds (P25, P75).

[b] $P < 0.05$ vs the wild - type group.

[c] $P < 0.05$ vs the heterozygote group.