Frequent SLC12A3 Mutations in Chinese Gitelman Syndrome Patients: Structure and Function Disorder

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Abstract

**Purposes:** This study was conducted to identify the frequent mutations from reported Chinese Gitelman syndrome (GS) patients, to predict three-dimensional structure change of human Na-Cl co-transporter (hNCC), and to test the activity of these mutations and some novel mutations *in vitro* and *in vivo.*

**Methods:** *SLC12A3* gene mutations in Chinese GS patients previously reported in the PubMed, CNKI and Wanfang database were summarized. Predicted configurations of wild type (WT) and mutant proteins were achieved using the I-TASSER workplace. Six missense mutations (T60M, L215F, D486N, N534K, Q617R and R928C) were generated by site-directed mutagenesis. $^{22}$Na$^+$ uptake experiment was carried out in the Xenopus laevis oocyte expression system. 35 GS patients and 20 healthy volunteers underwent the thiazide test.

**Results:** T60M, T163M, D486N, R913Q, R928C and R959frameshift were frequent *SLC12A3* gene mutations (mutated frequency >3%) in 310 Chinese GS families. The protein’s three-dimensional structure was predicted to be altered in all mutations. Compared with WT hNCC, the thiazide-sensitive $^{22}$Na$^+$ uptake was significantly diminished for all 6 mutations: T60M 22±9.2%, R928C 29±12%, L215F 38±14%, N534K 41±15.5%, Q617R 63±22.1% and D486N 77±20.4%. In thiazide test, the net increase in chloride fractional excretion in 20 healthy controls was significantly higher than GS patients with or without T60M or D486N mutations.

**Conclusions:** Frequent mutations (T60M, D486N, R928C) and novel mutations (L215F, N534K and Q617R) lead to protein structure alternation and protein dysfunction verified by $^{22}$Na$^+$ uptake experiment in vitro and thiazide test on patients.
Introduction

Gitelman syndrome (GS, OMIM263800) is a recessively inherited salt-losing tubulopathy caused by mutations of \textit{SLC12A3} gene, which encodes the thiazide-sensitive human Na-Cl co-transporter (hNCC NM_000339.2; OMIM 600968)\cite{1,2}. More than 500 \textit{SLC12A3} gene mutations were found previously (http://www.hgmd.cf.ac.uk/ac/index.php), and some frequent mutations were identified in Chinese, Japanese and European patients\cite{3-9}. Different frequent mutations of different populations indicate location and ancestral diversity of \textit{SLC12A3} gene mutation\cite{3,8}. In different studies of Chinese GS patients\cite{3,4,6,10,11}, T60M, D486N, R913Q, and R928C were reported as the frequent mutations, which were consistent with our previous studies\cite{12-18}. Even though the functional impact of mutations on NCC proteins could be confirmed in the \textit{Xenopus laevis oocyte} expression system \textit{in vitro}\cite{1,7,19-23}, GS mimic mouse models\cite{24,25} and thiazide test\cite{13,26,27,17} \textit{in vivo}, the functional characteristics of the most frequent NCC mutations and novel mutations of Chinese patients remain unknown. Few study integrate the protein configurations with the function of hNCC mutations \textit{in vitro} and \textit{in vivo}. Herein, this study was conducted to summarize all reported \textit{SLC12A3} gene mutations in Chinese GS patients and our 105 cases, to identify the most frequent ones, to predict the protein configurations, and to test the activity of these mutations \textit{in vitro} and \textit{in vivo}.

Materials and Methods

The study protocol was approved by the Ethics Committee on Human Studies at Peking Union Medical College Hospital (PUMCH), Chinese Academy of Medical Sciences, Beijing, China. The authors adhered to the Declaration of Helsinki, and patients of our hospital were included after providing informed consent.

Patient Recruitment and Mutation Analysis of \textit{SLC12A3} Gene

This study was based on the GS cohort that reported in our previous studies\cite{12-18}. From 2004, hypokalemic patients who presented to Peking Union Medical College Hospital with potassium loss from the kidney, metabolic
alkalosis, normotension were included. *SLC12A3* gene screening was performed to confirm the diagnosis of GS.

The method of *SLC12A3* gene direct sequencing was elaborated in our previous studies[12,13]. The most frequent mutations of the *SLC12A3* gene in Chinese patients were identified as follows. PubMed, China National Knowledge Infrastructure (CNKI) and Wanfang databases (Academic Search Engines for Chinese manuscripts) were searched with the keyword “Gitelman Syndrome” up to August 2019, and all the literature published by Chinese researchers (with individual patient’s gene mutations available) were included. The same patient repeatedly reported in different articles by the same group was only counted once. Those studies included apparent mistakes without reasonable explanation were excluded. Together with the mutations found in our laboratory, we calculated the type and number of mutated alleles. To avoid repeat calculations, a heterozygous mutated allele that occurred in one family was counted only once, a homozygous mutated allele was counted twice. We defined the mutated allele, whose frequency was greater than 3% as a frequent mutation.

*Configuration Prediction of WT and Mutant hNCC Proteins*

Predicted three-dimensional structures of WT and mutant proteins were achieved using the iterative threading assembly refinement (I-TASSER) workplace ([https://zhanglab.ccmb.med.umich.edu/I-TASSER/](https://zhanglab.ccmb.med.umich.edu/I-TASSER/))[28,29]. WT and 9 mutant protein amino acid sequences (T60M, T163M, L215F, D486N, N534K, Q617R, R913Q, R928C and R959frameshift) were sent to I-TASSER. The I-TASSER system uses C-Score to evaluate the accuracy of the models, C-score is typically in the range of [-5,2], with a higher value signifies a model with more confidence and vice-versa[28]. The model with highest C-score was chosen. The effect of mutations on protein configurations of NCC was visualized by PyMOL Viewer.

*Construction of WT and Mutated hNCC cDNA*

Human renal total RNA was isolated from the paracancerous tissue collected from patients undergoing nephrectomy due to renal cancer by TRizol® RNA extraction method (Life Technologies, the US). The first
strands cDNA was generated according to the manuscript of the Reverse Transcription System (Promega, the US). The hNCC cDNA was obtained by PCR with the forward primer 5'-ATGGCAGAACTGCCCACAACAGAC-3' and the reverse primer 5'-TTACTGGCAGTAAAAGGTGAGCACG-3'. Then, the hNCC cDNA was cloned into a PGEM-T vector (Promega, the US). Three frequent mutations (T60M, D486N, and R928C) and 3 novel mutations (L215F, N534K, and Q617R) were introduced into the hNCC-pGEM T vector by site-directed mutagenesis kit (TransGene, China). The WT and mutant hNCC cDNA pGEM T vectors were confirmed by DNA direct sequencing.

Xenopus laevis oocyte transport assay

As previous studies described[30-33], human SLC12A3 cRNA mutant variants were prepared by in vitro transcription reaction utilizing the T7 or SP6 mMessage mMachine (Ambion). Freezing X. laevis oocytes (stages V and VI) were obtained from the lab of National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health (the US). They were transferred to calcium-containing OR-2 (1 mM CaCl$_2$), and maintained at 18-20 °C until injection with cRNA. Oocytes were injected utilizing a Nanoject II injector (Drummond Scientific). Injection volumes were 36.8 nl, and cRNA concentrations 1 ng/ml. Sham-injected oocytes were injected with 36.8 nl of water. After injection oocytes were maintained in calcium-containing OR-2 at 18-20 °C until experiments were performed.

Three days post-injection, oocytes were transferred to Cl$^-$-free ND96 medium (96mM Na$^+$-isethionate, 2mM K$^+$-gluconate, 1.8mM Ca$^{2+}$-gluconate, 1mM Mg$^{2+}$-gluconate, 5mM Hepes, pH 7.4, 2.5mM sodium pyruvate and 5mg/100ml gentamicin) 24 hours. To begin uptake experiments, oocytes were incubated in Cl$^-$-free ND96 medium containing 1mM ouabain, 0.1mM amiloride and 0.1mM bumetanide for 30 min, following specified times (0.5~2 h) of uptake in K$^+$-free, NaCl medium (40mM NaCl, 56mM sodium-glutonate, 4mM CaCl$_2$, 1mM MgCl$_2$ and
5mM Hepes/Tris, pH 7.4) containing 1mM ouabain, 0.1mM amiloride, 0.1mM bumetanide and 1µCi/ml $^{22}\text{Na}^+$. After incubation at room temperature, oocytes were washed four times with ice-cold phosphate-buffered saline. Individual oocytes per replicate were solubilized with 10% SDS, and internalized radioactivity was quantified by Automatic Gamma Counter (Perkin Elmer) as nmol/oocyte. Each data point represents the mean value of 10-15 oocytes. Each experiment was repeated a minimum of 3 times with similar results.

**Thiazide test**

Thiazide test was performed according to the standard protocol as previously described[17,14,13,18]. Thirty-five of our 105 GS patients signed the consent form and participated the thiazide test. Twenty healthy volunteers underwent the thiazide test as well.

**Statistical Analysis**

Normally distributed variables were expressed as the mean ± SD and compared using unpaired t-tests. One-way analysis of variance was performed to evaluate the differences among 3 subgroups of GS patients, followed by least significant difference post hoc test to test. The differences of thiazide test between healthy volunteers and each subgroup of GS patients were compared using unpaired t-tests. Differences were considered significant when $P<0.05$. All statistical analyses were performed with the statistical software 17.0 (SPSS, Chicago, IL).

**Results**

**The Frequent Mutations of Chinese GS Patients**

A total of 105 GS patients from 101 non-consanguineous Chinese families in PUMCH were recruited in this study. Sixty-nine mutations, including 20 novel mutations, were identified in this cohort. In total, 83 papers were utilized in this study (supplemental table 1). As shown in figure 1, 155 $SLC12A3$ gene mutations were detected in 338 Chinese GS patients from 310 unrelated families, including 112 missense mutations, 9 nonsense mutations,
11 splicing mutations, 16 small deletions, 3 small insertions, 3 small indels and 1 gross deletion. One patient carried five mutant sites (1 homozygous nonsense mutation and 3 heterozygous missense mutations). Five patients, including 2 patients with two homozygous mutations and 3 patients with one homozygous mutation and 2 heterozygous mutations, carried four mutant sites. Twenty-seven patients carried three mutant sites. One hundred and seventy-three patients carried compound heterozygous mutations. Fifty-six patients carried homozygous mutations. Seventy-six patients carried single heterozygous mutations.

Figure 2 showed the frequency and distribution of the 155 mutations. The mutations were distributed in 25 of 26 exons and their flanking intronic regions (except exon 19). T60M, T163M, D486N, R913Q, R928C and R959frameshift were found to be the frequent mutations (mutated allele frequency greater than 3%). T60M was the most frequent mutation in Chinese GS patients, with 75 mutated alleles (12.7%). The second most frequent mutation was D486N detected in 52 of all 591 mutated alleles (8.8%), followed with the mutant alleles of R913Q(3.9%), R928C (3.6%), T163M (3.2%) and R959frameshift (3.2%) . These 6 mutations accounted for 35.4% of all 591 mutated alleles.

Configuration Prediction of WT and 9 Mutant hNCC Proteins

The C-score results of 5 models of WT NCC and 9 mutations predicted by I-TASSER system were shown in supplemental table 2. The configuration of 6 frequent mutations (T60M, T163M, D486N, R913Q, R928C and R959frameshift) were presented in figure 3, and the predicted structure of 3 novel mutations found in our early admitted GS patients (L215F, N534K and Q617R) were shown in supplemental figure 1. In the WT configuration, there was no $\beta$-sheet structures, which were found in the C terminus of all mutations. The predicted secondary structure of the 60th residue was changed from loop of WT threonine (figure 3A) to an $\alpha$-helix of methionine mutation (figure 3A'), similar in the 215th residue leucine mutated to phenylalanine (supplemental figure 1A & 1A'). When the 163th residue threonine mutated to methionine, the end of first transmembrane $\alpha$-helix changed
from A166 to Q165, and the beginning of the second transmembrane α-helix transformed from V169 to 1168 subsequently (figure 3B & 3B'). On the WT hNCC protein configuration, 913th residue arginine was between 2 α-helices. When it mutated to glutamine, it was located between a β-sheet and an α-helix (figure 3D & 3D').

R959frameshift was the mutation effect of c.2877_2878delAG, and this mutation was predicted to change the amino acids 959-968 and result in a premature stop codon at amino acid 969, leading to a truncated protein (figure 3F & 3F').

22Na+ Uptake Activity of 6 Missense Mutations

We selected 3 missense mutations of the 6 frequent mutations, and 3 novel mutations detected in our early admitted GS patients to test the 22Na+ uptake activity. The sequence of 6 missense mutations (T60M, L215F, D486N, N534K, Q617R and R928C) were presented in figure 4. The localization of each selected mutation on the predicted topology of hNCC was shown in figure 5A. T60M was an important phosphorylation site located within the N-terminus, L215F was positioned on the edge of the third transmembrane segment, D486N was located in the fourth intracellular loop, N534K was a transmembrane mutation, and Q617R and R928C were located in the C-terminus.

22Na+ uptake rates were demonstrated in figure 5B and 5C. WT hNCC transported 22Na+ robustly, whereas sham injections lacked activity. In comparison with WT hNCC (100%±12.6%), the thiazide-sensitive 22Na+ uptake was significantly diminished for all mutants (T60M 22±9.2%, R928C 29±12%, L215F 38±14%, N534K 41±15.5%, Q617R 63±22.1% and D486N 77±20.4%) (% of the 22Na+ transport capacity of WT) (figure 5B).

Furthermore, 100 µM metolazone (a hNCC blocker) inhibited the uptake of 22Na+ in wild-type and mutant NCC-expressing oocytes to background levels observed in sham injections (data was not shown). The rates of NCC mutants-mediated 22Na+ uptake were lower than 22Na+ transport capacity of WT at every time point (0.5~2 h), but the metolazone-sensitive 22Na+ uptake by WT and NCC mutants were all linear up for 2h of incubation (figure
Thiazide test

The thiazide test result were presented in figure 6, the net increase in chloride fractional excretion (FECl) in 20 healthy controls of our previous study was 4.46±1.04% [13, 17], which was significantly higher than the 3 subgroups of GS patients. The net increase in FECl after thiazide application in 3 GS patients with T60M mutation was 2.07±0.62%, in 7 GS patients with D486N mutation was 1.13±1.19%, and in 25 GS patients without any T60M or D486N mutation was 0.92±1.09%. No apparent difference was found among the subgroups.

Discussion

In this study, among 338 Chinese GS patients from 310 unrelated families, T60M, T163M, D486N, R913Q, R928C and R959frameshift were proved as the frequent SLC12A3 gene mutants with altered protein’s three-dimensional structure. Notable dysfunction of the mutated hNCC protein was confirmed by $^{22}\text{Na}^+$ uptake experiment carried out in the *Xenopus laevis* oocyte expression system and thiazide test in GS patients. It was the first time integrating the genetic mutation, hNCC protein structure and function *in vitro* and *in vivo*, which might facilitate the understanding of the genetic features of Chinese GS patients, as well as correlate genotype with phenotype of GS.

Till now, more than 500 SLC12A3 gene mutations have been found in GS patients of different ethnicity. In this study, we proved that T60M, D486N, T163M, R913Q, R928C and R959frameshift were the frequent SLC12A3 gene mutations in the largest sample of Chinese GS patients (n=338), published by Chinese investigators on PubMed and Chinese databases. It was consistent with the results of several previous studies, including the mutations of T60M, T163M, D486N, S710X, R871H, R913Q, R928C, R959frameshift, IVS13-191C>T and IVS21+253C>T (table 1) [3,10,6,4,11,18]. T60M, L858H and R642C were found to be the frequent mutations in Japanese articles [8,9]. Eight frequent mutations- L272P, A313V, c.1180+1G>T, G741R, L859P,
R861C, c.2883+1G>T, and C994Y - were identified in two large genetically diagnosed European GS patients cohorts [5,7]. None of European GS patients’ frequent mutations were identical with the hotspot mutations in Chinese or Japanese GS patients, indicating that the distribution of SLC12A3 gene mutations may differ from the location and ethnicities.

To our limited knowledge, we first integrally predicted the configuration alternation and investigated function change of the frequent mutations in the SLC12A3 gene. Both the change of secondary structure and three-dimensional structures were predicted to be altered in four frequent mutations (T60M, T163M, R913Q and R959frameshift), as well as in the novel mutation - L215F. The visible differences in the whole protein configuration caused by base substitution or bases deletion indicated these mutation were pathogenic. Among the 6 frequent mutations, T60 was an important phosphorylation site, and was very important for the membrane expression of hNCC and phosphorylation of the adjacent T46 and T55 sites [24]. D486N was located in the fourth intracellular loop, and the mutation effect was not well-studied. R928C (SNP rs12708965) was reported as a polymorphism, but it was believed to be deleterious and considered disease-causing [34]. The sodium and chloride transport ability of these mutant hNCC proteins remains to be determined. Herein, we constructed six missense mutant hNCC, including 3 frequent mutations - T60M, D486N and R928C, and 3 novel mutations - L215F, N534K and Q617R, and assessed their function status directly by \( \text{Na}^+ \) uptake experiments on the Xenopus laevis oocyte expression system in vitro. Compared with WT hNCC protein, \( \text{Na}^+ \) uptake capacity of the 6 mutant proteins varied from 22% to 77%, which may explain diversity in clinical presentation in GS patients. Regarding the in vitro functional study of NCC, a few NCC variants were studied by Xenopus laevis oocyte system previously [7,19-22].

As a monogenic disease, many studies tried to correlate phenotype to genotype [10,6,35,3,1,12]. Riveira-Munoz, E, et al reported that genotype and gender may determine the clinical severity in their cohort of GS patients.
In addition, our previous study found that serum magnesium level may indicate the severity of clinical presentation[12]. T60M and D486N were top 2 hotspot mutations in Chinese GS patients, and the result of \textit{in vitro} experiment illustrated apparent function difference between them. With regard to the \textit{in vivo} functional investigation of NCC, two mouse models were generated to mimic the GS pathophysiological procedure[24,25], and the thiazide test also determined the patient’s NCC function status directly[27,36,37,26,13,14,17,38]. Herein, we compared the thiazide test result among our GS patients with or without T60M or D486N mutations. However, till now, no obvious difference was observed among the 3 subgroups of GS patients. The inconsistency between \textsuperscript{22}Na\textsuperscript{+} uptake experiment \textit{in vitro} and thiazide test \textit{in vivo} may be caused by small sample size, limitation of \textit{in vitro} experiment and genetic heterogeneity. The expression and function of hNCC protein may be influenced by epigenetic modifications and silent polymorphisms[1]. More severe phenotype was observed in male patients compared with their female siblings who carried the same mutations[1,35,39]. However, no significant difference was found in thiazide test result between male and female GS patients in this study. T60M carriers in Han populations have markedly lower blood pressure and slightly higher fasting plasma glucose compared with normal controls[40].

Although we tried to summarize all reported Chinese GS patients’ mutations, there were still three studies excluded due to the individual mutations unavailable[4,6,41], this result may cause deviation in the calculation of mutation frequency. In addition, 76 of 338 Chinese GS patients (22.5%) only detected single heterozygous mutation, and the percentage of single heterozygous mutation patient in other large cohort studies varied from 9.4% to 22.6% [11,7,6,5]. More mutations may be detected if multiplex ligation-dependent probe amplification (MLPA) can be used to search for large rearrangements. Whether there is clinical presentation difference between these single heterozygous mutation GS patients and other GS patients need further study. More GS patients with only
T60M or D486N mutation participating the thiazide test may help us to better understand phenotype-genotype correlation of this disease.

In conclusion, we first integrally proved the protein structure alternation, as well as $^{22}\text{Na}^+$ uptake experiment in vitro and thiazide test on patients verified the dysfunction of mutated hNCC proteins, in Chinese GS patients with frequent mutations (T60M, D486N, R928C) and novel mutations (L215F, N534K and Q617R). Future studies are needed to reveal the underlying pathogenic mechanism in GS, and evaluate phenotype-genotype correlations to improve the prognosis of GS.

**Declaration of Interest**

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Lanping Jiang, Xiaoyan Peng, Binbing Zhao, Lei Zhang and Lubing Xu. The first draft of the manuscript was written by Lanping Jiang, and the manuscript was reviewed and edited by Xiaoyan Peng, Min Nie and Limeng Chen. All authors read and approved the final manuscript.

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References


18. Jiang, L.: Establishment of Gitelman Syndrome’s Diagnosis Methods and A Preliminary Study of Vitamin D’s Inhibition Mechanism on Renin Activation. Peking Union Medical College Hospital, Peking Union Medical College, Chinese Academy of Medical Sciences (2015)


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356 D.H.: Defective processing and expression of thiazide-sensitive Na-Cl cotransporter as a cause of
359 Gamba, G.: Pathophysiology of functional mutations of the thiazide-sensitive Na-Cl cotransporter in
361 doi:10.1152/ajprenal.00044.2004
22. Moreno, E., Tovar-Palacio, C., de los Heros, P., Guzman, B., Bobadilla, N.A., Vazquez, N., Riccardi, D.,
362 Poch, E., Gamba, G.: A single nucleotide polymorphism alters the activity of the renal Na+:Cl-
363 cotransporter and reveals a role for transmembrane segment 4 in chloride and thiazide affinity. The
367 24. Yang, S.S., Fang, Y.W., Tseng, M.H., Chu, P.Y., Yu, I.S., Wu, H.C., Lin, S.W., Chau, T., Uchida, S., Sasaki,
25. Yang, S.S., Lo, Y.F., Yu, I.S., Lin, S.W., Chang, T.H., Hsu, Y.J., Chao, T.K., Sytwu, H.K., Uchida, S., Sasaki,
370 S., et al.: Generation and analysis of the thiazide-sensitive Na+–Cl– cotransporter (Ncc/Slc12a3)
371 Ser707X knockin mouse as a model of Gitelman syndrome. Hum. Mutat. 31(12), 1304-1315 (2010).
372 doi:10.1002/humu.21364
375 doi:10.2215/cjn.02950906
27. Colussi, G., Rombola, G., Brunati, C., De Ferrari, M.E.: Abnormal reabsorption of Na+/Cl- by the thiazide-
376 inhibitable transporter of the distal convoluted tubule in Gitelman's syndrome. American journal of
377 nephrology 17(2), 103-111 (1997).
378 structure, sequence and protein-protein interaction information. Nucleic acids research 45(W1), W291-
381 Concentrations Induce Red Blood Cell Frailty: A Link to Diabetes Via Glucose, Glucose Transporters,
31. Amir Shaghaghi, M., Zhouyao, H., Tu, H., El-Gabalawy, H., Crow, G.H., Levine, M., Bernstein, C.N., Eck,
383 P.: The SLC2A14 gene, encoding the novel glucose/dehydroascorbate transporter GLUT14, is associated
385 doi:10.3945/ajcn.116.147603
386 H., Wang, Y., et al.: Vitamin C transporter Slec23a1 links renal reabsorption, vitamin C tissue
387 accumulation, and perinatal survival in mice. The Journal of clinical investigation 120(4), 1069-1083
388 (2010). doi:10.1172/jci39191
33. Soreq, H., Seidman, S.: Xenopus oocyte microinjection: from gene to protein. Methods Enzymol 207, 225-


Figure Legend

Figure 1. Number of different mutation types found in Chinese GS patients (A) and the number of mutant sites detected in each patient (B).

A. Total 155 $SLC12A3$ gene mutations were detected in 338 Chinese GS patients from 310 unrelated families, including 112 missense mutations (72.3%), 9 nonsense mutations (5.8%), 11 splicing mutations (7.1%), 16 small deletions (10.3%), 3 small insertions (1.9%), 3 small indels (1.9%) and 1 gross deletion (0.6%).

B. Five mutant sites were detected in 1 patient, 4 mutant sites were found in 5 patients, 3 mutant sites were carried by 27 patients, 2 mutant sites (compound heterozygous, CH) were detected in 173 patients, 2 mutant sites (homozygous, Homo) were found in 56 patients, and single heterozygous mutation were carried by 76 patients.

Figure 2. Frequency and distribution of the 155 detected mutations and 591 mutated alleles.

Total 338 Chinese patients from 310 unrelated families were diagnosed by $SLC12A3$ gene sequencing, and 155 mutations and 591 mutated alleles were detected in these families. T60M, T163M, D486N, R913Q, R928C, R959frameshift were the most frequent mutations (mutated allele frequency greater than 3%). On the horizontal axis, each bar represents one mutation (there is no relationship with the actual position in the exon). The dotted line corresponds to an allele frequency of 3% ($n=17.73$).

Figure 3. Predicted configuration of WT and 6 frequent mutant hNCC proteins.

Figures A-F are configurations of WT hNCC proteins with local amplification of T60 (A), T163 (B), D486 (C), R913 (D), R928 (E) and R959 (F). Figure A’-F’ are the configurations of corresponding mutant hNCC proteins with local amplification of M60 (A’), M163 (B’), N486 (C’), Q913 (D’), C928 (E’) and 959frameshift (F’).

Figure 4. DNA sequence results of 6 mutated hNCC-pGEM T vectors.
The DNA sequence results of 6 mutated hNCC-pGEM T vectors (T60M, L215F, D486N, N534K, Q617R and R928C). The mutations’ sequence results were listed at the left column, and the corresponding sites of WT were presented at the right column. The sites of mutations denoted by red arrows.

**Figure 5** Location of the 6 studied *SLC12A3* mutations in the predicted hNCC protein and the result of $^{22}\text{Na}^+$ uptake experiment.

A. The schematic topological representation of NCC consists of the intracellular N- and C-terminal domains and 12 transmembrane segments. We studied the function of the mutations labeled T60M, L215F, D486N, N534K, Q617R and R928C. B. Metolazone-sensitive $^{22}\text{Na}^+$ uptake was measured in oocytes injected with H$_2$O, WT (open bars) or mutant *SLC12A3* cRNAs (black bars). The uptake values were shown as percentages of WT $^{22}\text{Na}^+$ transport (WT was set as 100%). C. Time course of $^{22}\text{Na}^+$ uptake in WT and hNCC mutant-injected *X. laevis* oocytes. *X. laevis* oocytes were microinjected with the following *SLC12A3* cRNAs: WT, sham, and mutants T60M, L215F, D486N, N534K, Q617R and R928C. Data were presented as the mean±SEM, and compared using unpaired t-tests. *P<0.05 indicated a significant difference compared with WT *SLC12A3*-injected oocytes. Each data point represents the mean value of 10-15 oocytes.

**Figure 6.** The thiazide test result verified hNCC dysfunction in GS patients, while no apparent difference was found among the GS patients with or without T60M or D486N mutation.

The net increase in chloride fractional excretion (FECI) undergoing thiazide test in 20 healthy controls (4.46±1.04 %), 3 GS patients with T60M mutation (2.07±0.62 %), 7 GS patients with D486N mutation (1.13±1.19 %), and 25 GS patients without any T60M or D486N mutation (0.92±1.09 %). One-way analysis of variance was performed to evaluate the differences among 3 subgroups of GS patients, followed by least significant difference post hoc test to test. The differences of thiazide test between healthy volunteers and each subgroup of GS patients...
were compared using unpaired t-tests.

**Supplemental figure 1. Predicted configuration of WT and 3 novel mutant hNCC proteins**

Figures A-C are configurations of WT hNCC protein with local amplification of L215 (A), N534 (B), Q617 (C).

Figure A′-C′ are the configurations of corresponding mutant hNCC proteins with local amplification of F215 (A′), K534 (B′) and R617 (C′).
A

- Missense=112
- Nonsense=9
- Splicing=11
- Small deletions=16
- Small insertions=3
- Small indels=1
- Gross deletions=1
Total=155

B

- 5 mutant sites=1
- 4 mutant sites=5
- 3 mutant sites=27
- 2 mutant sites (CH3)=173
- 2 mutant sites (Homo)=56
- 1 mutant site=76
Total=338
<table>
<thead>
<tr>
<th>Author [Reference number]</th>
<th>Number of GS patients</th>
<th>Frequent mutations</th>
<th>Country or district (published year)</th>
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<tbody>
<tr>
<td>This study</td>
<td>310 families</td>
<td>T60M, T163M, D486N, R913Q, R928C, R959frameshift</td>
<td>China</td>
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</tbody>
</table>
# Supplemental table 1: Eighty three papers utilized in the manuscript to find out the most frequent

**SLC12A3** gene mutations in Chinese GS patients with blood pressure.

<table>
<thead>
<tr>
<th>Title</th>
<th>Family number</th>
<th>Patient number</th>
<th>Blood pressure (BP, mmHg)</th>
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<td>Clinical Severity of Gitelman Syndrome Determined by Serum Magnesium¹</td>
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<td>105(45)</td>
<td>110.0 ± 12.4/72.1 ± 9.8</td>
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<td>Normomagnesemic Gitelman Syndrome Patients Exhibit a Stronger Reaction to Thiazide Than Hypomagnesemic Patients²</td>
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<td>Value of Chloride Clearance Test in Differential Diagnosis of Gitelman Syndrome³</td>
<td></td>
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<tr>
<td>Increased urinary prostaglandin E2 metabolite: A potential therapeutic target of Gitelman syndrome⁴</td>
<td></td>
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<td>Glucose tolerance and insulin responsiveness in Gitelman syndrome patients⁵</td>
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<tr>
<td>Hydrochlorothiazide Test as a Tool in the Diagnosis of Gitelman Syndrome in Chinese Patients⁶</td>
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<tr>
<td>Establishment of Gitelman Syndrome’s Diagnosis Methods and A Preliminary Study of Vitamin D’s Inhibition Mechanism on Renin Activation⁷</td>
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<td>Identification of five novel variants in the thiazide-sensitive NaCl co-transporter gene in Chinese patients with Gitelman syndrome⁸</td>
<td>34</td>
<td>41(14)</td>
<td>107 ± 10/ 70 ± 9</td>
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<td>Novel SLC12A3 mutations in Chinese patients with Gitelman's syndrome⁹</td>
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<td></td>
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<tr>
<td>The Study of Molecular Mechanism and Clinical Analysis of Gitelman's Syndrome¹⁰</td>
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<td>Phenotype of Chinese patients with Gitelman syndrome and the effect of gender on the phenotype¹¹</td>
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<td>Identificaton and functional analysis of mutations of SLC12A3 gene in Chinese patients with Gitelman syndrome¹²</td>
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<td></td>
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<tr>
<td>Abnormal glucose metabolism and insulin sensitivity in Chinese patients with Gitelman syndrome¹³</td>
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<tr>
<td>Characteristics and Follow-Up of 13 pedigrees with Gitelman syndrome¹⁴</td>
<td>13</td>
<td>17(8)</td>
<td>106.6 ± 4.1/ 68.3 ± 5.5</td>
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<td>Identification of two novel mutations in SLC12A3 gene in two Chinese pedigrees with Gitelman syndrome and review of literature¹⁵</td>
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<td>Clinical and genetic characteristics of Gitelman syndrome in 5 pedigrees¹⁶</td>
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<td>Genetic Features of Chinese Patients with Gitelman Syndrome: Sixteen Novel SLC12A3 Mutations Identified in a New Cohort¹⁷</td>
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<td>Male:117.7 ± 9.5/73.1 ± 7.1</td>
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<td>Female: 108.5 ± 10.0/69.4 ± 5.2</td>
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<td>Hypokalemic paralysis due to Gitelman syndrome: a family study¹⁸</td>
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<td>A novel SLC12A3 splicing mutation skipping of two exons and preliminary screening for alternative splice variants in human kidney¹⁹</td>
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<td>Family paralysis²⁰</td>
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2 patients'
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<td>Phenotype and genotype analysis in Chinese patients with Gitelman's syndrome</td>
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<td>Coexistence of Gitelman's syndrome and thyroid disease: SLC12A3 gene analysis in two patients</td>
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<td>10(3) 114.0 ± 11.0/73.5 ± 6.7</td>
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<td>Analysis of SLC12A3 gene mutation in patients with Gitelman syndrome</td>
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<td>6(4) 117.8 ± 14.7/70.5 ± 5.8</td>
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<td>7(3) 113.3 ± 15.5/69.9 ± 7.6</td>
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<td>Clinical feature and genetic analysis of Gitelman's syndrome accompanied by autoimmune thyroid disease</td>
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<td>Experience in Clinical diagnosis and treatment of refractory Gitelman syndrome: a case report</td>
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<td>Gitelman syndrome in children: report of two cases and literature review</td>
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## References


7. Jiang, L: Establishment of Gitelman Syndrome’s Diagnosis Methods and A Preliminary Study of Vitamin D’s Inhibition Mechanism on Renin Activation. *Department of Nephrology*. Peking Union Medical College Hospital, Peking Union Medical College, Chinese Academy of Medical


24. Dong, H, Lang, Y-h, Shao, Z-p, Li, L, Shao, L-p: Coexistence of Gitelman's syndrome and thyroid


40. Yang, Y, Li, H, Shi, LX: Clinical characteristics of a case of Gitelman syndrome and the gene


Supplemental table 2. The C-score results of 5 models of wild type (WT) NCC and 9 mutations predicted by I-TASSER system.

<table>
<thead>
<tr>
<th></th>
<th>Model 1</th>
<th>Model 2</th>
<th>Model 3</th>
<th>Model 4</th>
<th>Model 5</th>
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<tr>
<td>WT</td>
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<td>-3.14</td>
<td>-3.13</td>
<td>-3.15</td>
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<tr>
<td>T60M</td>
<td>0.17</td>
<td>-1.07</td>
<td>-1.13</td>
<td>-1.61</td>
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<tr>
<td>T163M</td>
<td>-0.47</td>
<td>-0.86</td>
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<td>-1.56</td>
<td>-1.97</td>
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<tr>
<td>L215F</td>
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<td>-0.82</td>
<td>-1.91</td>
<td>-1.66</td>
<td>-2.39</td>
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<tr>
<td>D486N</td>
<td>-0.01</td>
<td>-0.71</td>
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<td>-1.47</td>
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<tr>
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<td>-1.06</td>
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<td>R913Q</td>
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<td>-1.68</td>
<td>-2.75</td>
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</tr>
</tbody>
</table>

Note: The I-TASSER system uses C-Score to evaluate the accuracy of the models, based on the significance of threading template alignments and the convergence parameters of the structure assembly simulations. C-score is typically in the range of [-5,2], with a higher value signifies a model with more confidence and vice-versa.