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Mutation Screening and Functional Study of *SLC26A4* in Chinese Patients with Congenital Hypothyroidism

Chang-Run Zhang*, Yuan-Ping Shi*, Cao-Xu Zhang, Feng Sun, Wen-Jiao Zhu, Rui-Jia Zhang, Ya Fang, Qian-Yue Zhang, Chen-Yan Yan, Ying-Xia Ying, Shuang-Xia Zhao, Huai-Dong Song

The Core Laboratory in Medical Center of Clinical Research, Department of Endocrinology, Shanghai Ninth People's Hospital, Shanghai JiaoTong University School of Medicine, Shanghai, China

**Authors contributed equally.*

What is already known on this topic?

Defects in the human *SLC26A4* gene are reported to be one of the causes of congenital hypothyroidism (CH). *SLC26A4* encodes the apical iodide transporter protein, pendrin.

What this study adds?

We identified seven distinct variants of *SLC26A4*, including one novel mutation, in a cohort of Chinese patients with CH. Functional studies showed that five out of six missense mutations had different effects on gene function, including ion transport and/or membrane location of the SLC26A4 protein, Pendrin. These results provide an important basis for future mechanism research.

Abstract

Objective: Defects in the human solute carrier family 26 member 4 (*SLC26A4*) gene are reported to be one of the causes of congenital hypothyroidism (CH). We aimed to identify *SLC26A4* mutations in Chinese patients with CH and analyze the function of the mutations.

Methods: Patients with primary CH were screened for 21 CH candidate genes mutations by targeted next-generation sequencing. All the exons and exon-intron boundaries of *SLC26A4* were identified and analyzed. The function of six missense mutation in *SLC26A4* were further investigated *in vitro*.

Results: Among 273 patients with CH, seven distinct *SLC26A4* heterozygous mutations (p.S49R, p.I363L, p.R409H, p.T485M, p.D661E, p.H723R, c.919-2A>G) were identified in 10 patients (3.66%, 10/273). *In vitro* experiments showed that mutation p.I363L, p.R409H, p.H723R affect the membrane location and ion transport of *SLC26A4*, while p.S49R did not. Mutation p.T485M and p.D661E only affected ion transport, but had no effect on the membrane location.

Conclusion: The prevalence of *SLC26A4* mutations was 3.66% in Chinese patients with CH. Five mutations (p.I363L, p.R409H, p.T485M, p.D661E and p.H723R) impaired the membrane location or ion transport function of *SLC26A4*, suggesting important roles for Ile363, Arg409, Thr485, Asp661, and His723 residues in *SLC26A4* function. As all variants identified were heterozygous, the pathogenesis of these patients cannot be explained, and the pathogenesis of these patients needs further study.

Keywords: Congenital hypothyroidism, next-generation sequencing, *SLC26A4*, cell location, ion transport

Introduction

Congenital hypothyroidism (CH) is a common neonatal endocrine disorder. Unless treated in the first few months of life, severe CH can lead to growth retardation and permanent intellectual disability (1). The incidence of CH was reported to be about 1:4000 in 1970, and the incidence of the disease has increased to 1:2000 in the past decades (2). About 85% of cases of CH are caused by abnormal thyroid development (thyroid dysgenesis), but genetic associations with thyroid dysgenesis have only been identified in 2-5% of cases. These pathogenic genes leading to dysgenesis include

Address for Correspondence: Huai-Dong Song MD, The Core Laboratory in Medical Center of Clinical Research, Department of Endocrinology, Shanghai Ninth People's Hospital, Shanghai JiaoTong University School of Medicine, Shanghai, China
Phone: +86 21 23271699 **E-mail:** huaidong_s1966@163.com **ORCID:** orcid.org/0000-0002-9609-5803

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thyroid stimulating hormone (TSH) receptor *(TSHR), PAX8, GLIS3, NKX2.1* and *FOXE1* (3). The remaining 15% of cases of CH are due to defects of thyroid hormone biosynthesis (dyshormonogenesis). Dyshormonogenesis is often caused by mutations in genes that are involved in the pathway of thyroid hormone synthesis, such as thyroperoxidase (TPO), dual oxidase 2 (DUOX2), sodium-iodide symporter (NIS; SLC5A5) and the apical iodide transporter, pendrin (PDS; *SLC26A4*) (4).

SLC26A4 encodes pendrin, a multi transmembrane (TM) protein composed of 780 amino acids, consisting of 12-14 TM segments and a segment of intracellular STAS (Sulfate Transporter and Anti-Sigma factor antagonist) functional domain (5,6,7,8,9). Pendrin is an anion exchanger that is highly expressed in thyroid, inner ear and kidneys. In thyroid, pendrin is expressed at the apical membrane of thyroid follicular cells. It acts as a chloride-iodide exchanger, transporting iodide from the cell to the follicular lumen, where thyroid hormone is synthesized (10). Although previous studies have shown that *SLC26A4* biallelic mutation may result in CH (11), biallelic mutation of *SLC26A4* have not been found in Chinese patients with CH. In order to evaluate the role of *SLC26A4* in the pathogenesis of the Chinese CH patients, our study identified *SLC26A4* mutations in a cohort of Chinese patients with CH and analyzed the function of any identified mutations *in vitro*.

Methods

Clinical Subjects

We enrolled Chinese CH patients through newborn screening. Newborn screening was done with filter-paper blood spots between 3 and 5 days after birth. Blood samples were collected from the heel and TSH level was measured by time-resolved fluorescence assay (PerkinElmer, USA). Subjects with increased TSH (TSH ≥10 mU/L) levels observed during neonatal screening were recalled for further evaluation. The levels of TSH, total triiodothyronine (T3), total thyroxine (T4), free T3, and free T4 (fT4) in serum were determined by performing an immuno-chemiluminometric assay (UniCel DxI 800, Beckman, USA). The details of the diagnostic criteria to establish permanent CH in patients were from our previous study (12); briefly these included i) elevated TSH levels, ii) T4 or fT4 levels less than the reference range, and iii) restoration of normal thyroid parameters after receiving replacement therapy with L-thyroxine, but, after stopping treatment, a rise in TSH and a drop in fT4 were observed again. In addition, some patients were recruited from outpatient clinics who were on L-thyroxine replacement therapy. Although these patients lack initial diagnostic data, they have a definite history of CH. A written consent was obtained from the parents of the CH patients, and the study was approved by the Ethics Committee of Shanghai Ninth People's Hospital affiliated to Shanghai JiaoTong University School of Medicine (decision no: 2016-76-T33, date: 2016-08-03). Informed consent was obtained from all patients or their legal guardians, and all unaffected family members who participated in the study.

Next-generation Sequencing

Genomic DNA was extracted from the peripheral blood using the Quick Gene DNA Whole Blood Kit L (Kurabo, Japan) according to the manufacturer's protocol (13). Twenty-one previously reported possible causative genes for CH, including TPO (GenBank reference sequence: NM_000547), SLC5A5 (NM_000453), thyroglobulin (TG) (NM_003235), TSHR (NM_000369), DUOX2 (NM_014080), DUOXA2 (NM_207581), *SLC26A4* (NM_000441), FOXE1 (NM_004473), PAX8 (NM_013952), NKX2-1 (NM_001079668), NKX2-5 (NM_004387), IYD (NM_001164694), DIO1 (NM_000792), DIO2 (NM_000793), THRA (NM_001190918), THRB (NM_00125263), DUOX1 (NM_017434), DUOXA1 (NM_001276268), GNAS (NM_016592), SLC16A2 (NM_006517) and HHEX (NM_002729) were analyzed in this study (16). All the exons and exon-intron boundaries of these genes were amplified by performing multiplex polymerase chain reaction (PCR) using a 48×48 Access Array™ microfluidic platform (Fluidigm, USA) according to the manufacturer's protocol. The primers were designed using iPLEX Assay Design software (Sequenom, USA). The HiSeq 3000 platform (Illumina, San Diego, CA, USA) was used to perform deep sequencing of these amplicon libraries. The target sequences were amplified and deep sequenced in duplicate for each sample to avoid base pair (bp) variants caused by multiplex PCR.

Calling of *SLC26A4* Variants from Next-generation Sequencing Data and Verification Using Sanger Sequencing

Raw sequence data was analyzed in fastq format and the quality scores were obtained, as previously described (14,15). Credible variants were selected according to the following criteria: (i) the quality scores of variants with ≥30 bps; (ii) mapping the quality scores of variants with ≥50 bps; (iii) sequencing to estimate the depth of variants with ≥20 bps; (iv) variant allele frequency ≥30%; (v) variants with read depth ≥5; and (vi) the presence of mutation on both the DNA strands (16). Variants with frequencies >1% in the dbSNP 135 and ESP6500 v2 databases were filtered out and the focus of the study was on the functional (protein altering) variants after removal of intergenic and 3'/5' UTR variants, nonsplice related intronic variants, synonymous

variants identified in duplicate samples. Then the remaining variants were selected for validation by Sanger sequencing.

Construction of Plasmid

Human wild-type (WT) cDNA of *SLC26A4* was cloned into p-enhanced green fluorescent protein (EGFP)-N2 plasmid (TransGen Biotech, China). Identified missense mutations were introduced into the *SLC26A4*-pEGFP-N2 WT plasmid by Fast Mutagenesis System kit (TransGen Biotech, China) according to the manufacturer's protocol. Meanwhile, human NIS cDNA was cloned into a eukaryotic expression vector pcDNA3.1. All the plasmid constructs were validated by Sanger sequencing.

Cell Culture and Transfections

293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/high-glucose medium (Gibco, USA) supplemented with 10% fetal bovine serum (Sigma Aldrich, USA) at 37 °C in a humidified atmosphere containing 5% CO_2. Transfections were performed on cells by Lipofectamine™ 2000 Transfection Reagent (Invitrogen™, USA) following the manufacturer's instructions. Cells were plated in 20 mm glass bottom cell culture dish (NEST), transfected with 1 μg plasmid DNA to detected the cell localization of the WT or mutants of *SLC26A4* plasmids. Iodide efflux assays were performed on 293T cells, cultured in 12 well plates, co-transfected with 0.5 μg pcDNA3.1-*NIS* and 0.5 μg WT or mutant *SLC26A4*-pEGFP-N2 plasmids.

The Assays for the Cell Localization of WT or Mutants of *SLC26A4*

Forty-eight hours after transfecting with WT or mutants of *SLC26A4*-pEGFP-N2 plasmids, 293T cells were washed twice in PBS (1X). Then cells were fixed in 4% paraformaldehyde for 30 minutes. After washing with PBS (1X), cells were stained with the membrane probe DiI (Beyotime, Haimen, China) at 37 °C for 5-10 min, then nucleii were stained with DAPI (Beyotime Biotech, Haimen, China) at room temperature for 5 minutes. Confocal imaging for cells was carried out on Nikon A1 confocal microscope using the 40x objective (Nikon A1 Microsystems, Japan).

Iodide Efflux Assay for WT or Mutants of *SLC26A4* in 293T Cells

The iodide efflux assay was performed as described previously (17). In brief, forty-eight hours after co-transfecting with 0.5 μg pcDNA3.1-*NIS* and 0.5 μg WT or mutant *SLC26A4*-pEGFP-N2 plasmids, 293T cells were washed once in serum-free DMEM medium and incubated for 1 hour in 1 mL serum-free medium containing ^{131}I at 5 KBq/mL as the only source of iodide. The cells were then washed briefly in HBSS buffer and then incubated with 1 mL HBSS for 5 minutes after which HBSS was removed. The cells were solubilized by the addition of 1 mL 1 N NaOH and the radioactivity measured using a γ counter (GC1200, Anhui, China). All experiments were carried out three times on triplicate cultures. Statistical significance of the iodide efflux assay results was determined by use of t-test.

Statistical Analysis

All data are expressed as mean ± standard deviation. Data analysis is mainly processed by Excel, elisacalc and Statistical Package for the Social Sciences 19.0 statistical software. $P < 0.05$ is considered to be statistically significant. The images in this paper are mainly processed and produced by Photoshop software, image J and Graphpad prism 6. The gene sequence retrieval website National Center for Biotechnology Information used in this paper: http://www.ncbi.nlm.nih.gov/; University of California, Santa Cruz: http://genome.ucsc.edu/

Results

Clinical Characteristics of Patients with CH

The cohort of CH patients enrolled consisted of 273 patients, including 141 (51.6%) females. The median value of serum TSH and serum fT4 level were 54.075 uIU/mL and 0.718 ng/dL, respectively. All of them had normal hearing. Whether these children had enlarged vestibular aqueduct (EVA) is unknown because the examination was unnecessary and the patient's family refused.

Screening the Missense Mutations of *SLC26A4* in the Chinese Patients with CH

All the exons and exon-intron boundaries were amplified by performing multiplex PCR using customized primers designed to generate 200-250bp amplicons. After the quality control assessment, the average coverage of *SLC26A4* with sequencing depth ≥20x was 89.04%. Seven heterozygous mutations in *SLC26A4* were identified in 10 patients, including one novel mutation (p.I363L). Interestingly, 8 of these 10 patients also carried mutations in other candidate gene for CH (Table 1). All mutation sites were verified by Sanger sequencing (Figure 1), with the exception of one patient. In patient 190 it was not possible to obtain a Sanger sequencing result because of DNA sample damage and patient refusal to provide a repeat sample. The frequency of *SLC26A4* mutation in Chinese patients diagnosed with CH was 3.66% (10/273). Among the seven mutations, p.S49R was located in the N-terminal intracellular region, p.D661E and p.H723R were located in the STAS domain of the C-terminal intracellular region which plays a key role in the membrane location of *SLC26A4*. The remaining mutation

Table 1. The clinical data and genetic characteristics of the 10 congenital hypothyroidism patients with mutation of *SLC26A4*

ID	Gender	At diagnosis				Mutation information				The frequency observed in public databases	
		Age (day)	Thyroid ultrasound	fT4 (0.58-1.64) ng/dL	TSH (0.34-5.6) uIU/mL	Mutated gene	Annotation	Zygosity	Classify sequence variants according ACMG/AMP guideline	ExAC_ ALL	ExAC_ EAS
6	Male	30	Thyroid ectopy	NA	43	*SLC26A4*	NM_000441:c.A2168G p.H723R	Heterozygous	Pathogenic	1.24E-04	0.0006
31	Female	30	Normal	NA	> 150	*SLC26A4*	NM_000441:c.A1087C p.I363L	Heterozygous	Uncertain significance	9.10E-05	4.40E-04
						TG	NM_003235:c.G5486C p.R1829P	Heterozygous	Uncertain significance	NA	NA
42	Male	30	Absence	NA	150	*SLC26A4*	NM_000441:c.C147G p.S49R	Heterozygous	Likely benign	0	0
						TG	NM_003235:c.A2276G p.Y759C	Heterozygous	Uncertain significance	1.70E-05	8.00E-05
						TG	NM_003235:c.C4859T p.T1620M	Heterozygous	Likely benign	0.0005	0.0068
51	Male	40	Goiter	> 0.4	> 100	*SLC26A4*	NM_000441:c.919-2A > G	Heterozygous-	NA	8.24E-06	0
						DUOX2	NM_014080:c.C4027T p.L1343F	Heterozygous	Pathogenic	7.42E-05	0.0003
						DUOX2	NM_014080:c.G2794A p.D932N	Heterozygous	Uncertain significance	2.49E-05	0
125	Male	15	NA	0.8	63.71	*SLC26A4*	NM_000441:c.C1983A p.D661E	Heterozygous	Likely benign	0.0001	0.0016
						DUOX2	NM_014080:c.C227T p.P76L	Heterozygous	Uncertain significance	3.43E-05	0.0005
190	Male	NA	NA	NA	NA	*SLC26A4*	NM_000441:c.C1454T p.T485M	Heterozygous	Uncertain significance	5.77E-05	0.0002
241	Female	30	Normal	0.65	> 150	*SLC26A4*	NM_000441:c.G1226A p.R409H	Heterozygous	Pathogenic	1.24E-04	0
						TSHR	NM_003235:c.T1574C p.F525S	Heterozygous	Uncertain significance	1.32E-04	1.74E-03
245	Male	20	Normal	1.41	20.48	*SLC26A4*	NM_000441:c.919-2A > G	Heterozygous	NA	8.24E-06	0
						DUOX2	NM_014080:c.A2033G p.H678R	Heterozygous	Benign	0.1020	0.055
						IYD	NM_001164694:c.793_794delTGinsCA p.C265H	Heterozygous	NA	NA	NA
247	Male	21	Normal	0.22	> 100	*SLC26A4*	NM_000441:c.919-2A > G	Heterozygous-	NA	8.24E-06	0
						DUOX2	NM_014080:c.G3616A p.A1206T	Homozygous	Pathogenic	7.41E-05	0
						IYD	NM_001164694:c.793_794delTGinsCA p.C265H	Heterozygous	NA	NA	NA
						TSHR	NM_003235:c.G1349A p.R450H	Heterozygous	Pathogenic	0.0003	0.0044
259	Female	40	Normal	0.53	51.676	*SLC26A4*	NM_000441:c.A1087C p.I363L	Heterozygous	Uncertain significance	9.10E-05	4.40E-04
						DUOXA2	NM_207581:c.554 + 6T > C	Homozygous	NA	0.8764	0.9352

Table 1. Continued

ID	Gender	At diagnosis				Mutation information				The frequency observed in public databases	
		Age (day)	Thyroid ultrasound	fT4 (0.58-1.64) ng/dL	TSH (0.34-5.6) uIU/mL	Mutated gene	Annotation	Zygosity	Classify sequence variants according ACMG/AMP guideline	ExAC_ ALL	ExAC_ EAS
						TG	NM_003235:c.G7318A p.V2440I	Heterozygous	Uncertain significance	5.80E-05	1.20E-04
						TG	NM_003235:c.A5791G p.I1931V	Heterozygous	Likely benign	0.0002	0.0021
						DUOX2	NM_014080:c.G2654T p.R885L	Heterozygous	Pathogenic	3.38E-04	4.39E-03

fT4: free thyroxine, TSH: thyroid-stimulating hormone, NA: not available

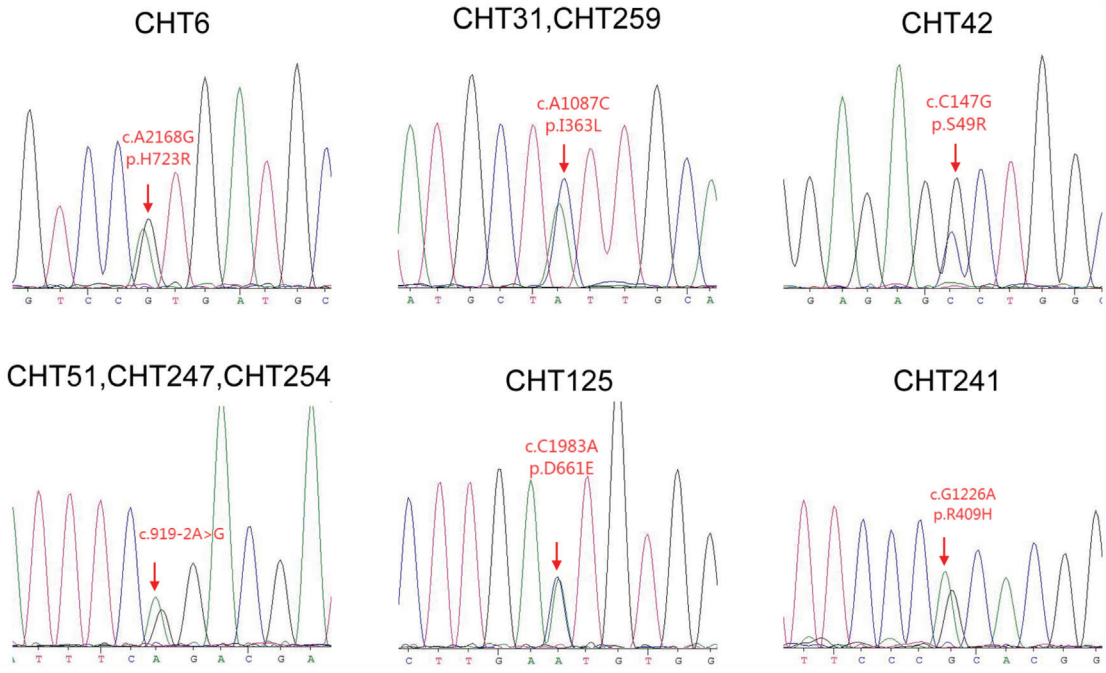

Figure 1. Sanger sequencing of *SLC26A4* mutation

sites were scattered in the 12 TM regions of *SLC26A4* (Figure 2).

Cellular Localization of *SLC26A4* Mutants

SLC26A4 has been shown, by immunohistochemical analysis, to be located at the apical membrane of thyroid follicular cells (18). To assess the effect of mutations on membrane location of *SLC26A4*, we expressed WT and mutants of the *SLC26A4*-pEGFP-N2 plasmid in 293T cells and observed location using a confocal fluorescence microscope. Although these cells lack the polarization of thyroid follicular cells, WT *SLC26A4* was clearly present at the cell membrane and significant co-localization with marker of cell membrane. Mutant p.S49R showed a cell membrane protein distribution similar to that of the WT *SLC26A4*. Mutants p.R409H and p.H723R did not express on the cell membrane obviously. Novel mutant p.I363L showed partly express in the cytoplasm, but was mostly expressed at the cell membrane. The remaining mutants, p.T485M, p.D661E, and p.S49R had no effect on cell membrane localization (Figure 3).

Assessment of Iodide Transport in the WT and Identified *SLC26A4* Mutants

It has been suggested that *SLC26A4* mediates iodide efflux at the apical membrane of thyroid follicular cell (10). In

order to assess the effect of mutations on the ability of iodide transport, we co-expressed NIS with WT or mutant *SLC26A4*-pEGFP-N2 plasmids in 293T cells to provide a cell model by which cells could uptake iodide from culture medium. The 293T cells transfected with *NIS* only showed an accumulation of radioiodide (^{131}I) in the cells. In contrast, only a small amount of ^{131}I was retained in the 293T cells after co-transfection with *NIS* and WT *SLC26A4* plasmids. However, compared to the 293T cells co-transfected with *NIS* and WT *SLC26A4* plasmids, with one exception (p.S49R), cells co-transfected with NIS and the identified *SLC26A4* mutants resulted in significantly decreased iodine efflux, indicating that these mutants lead to a decrease in cellular ability of iodide transport in 293T cells (Figure 4).

Discussion

CH is a relatively common endocrine disease with a prevalence ranging from 1:2000 to 1:4000 in newborns (19). Most cases are dysgenetic although a substantial proportion are due to dyshormonogenesis. There is a close association between genetic abnormalities and dyshormonogenesis but the search for genetic mechanisms in dysgenesis has identified <5% to have a genetic pathogensis. Therefore, it is important to expand the spectrum of the pathogenic genes in patients with CH, given the relatively common occurrence. In this study, mutations in *SLC26A4* were investigated in Chinese patients with CH and seven different heterozygous variants in 10 individuals (10/273, 3.66%) were found. The prevalence of *SLC26A4* mutations in our study was similar to a previous study that screened *SLC26A4* mutation in CH patients from Guangxi Zhuang Autonomous Region, China (20). Fu et al (20) reported that all the mutations detected were heterozygous mutations, and thus cannot be assumed to be pathogenetic. These findings suggested that *SLC26A4* might be an uncommon pathogenic gene for CH in the Chinese population.

SLC26A4 is a member of the SLC26 anion transporter family that encodes the pendrin protein which was originally predicted to contain 12 TM domains (21). This has since been shown to be incorrect as 14 TM domains were subsequently confirmed by Gorbunov et al (22). These regions contain many anion-binding sites or substrate-binding sites, including TM1, TM3, and TM10, which would affect the function of this protein (5). The protein also has a STAS domain in the cytosol, which is critical for membrane targeting of many SLC26 anion transporters, and STAS domain mutations are associated with at least three human recessive diseases (22). In our study, a total of seven mutation sites in *SLC26A4* were identified, including a novel variant which, to the best of our knowledge, has not previously been reported.

The variant S49R, located in the N-terminal intracellular segment, was shown by functional experiments to have no effect on membrane localization or ion transport. It may be that the site is not an ion binding site, so the mutation has little effect on gene function. The novel variant, I363L was located in TM8, which is an anion-binding domain (5). Our study confirmed that mutation I363L affects the membrane localization of *SLC26A4* slightly and reduced its ability to transport iodine ions by about 53%. We speculate that

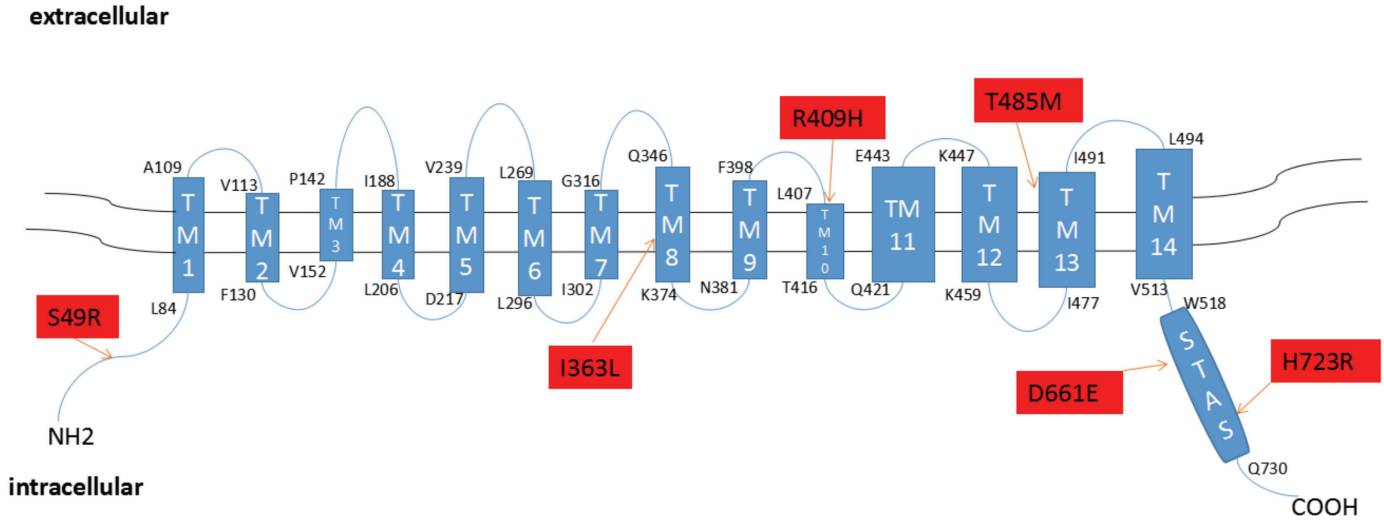

Figure 2. The mutations, identified from our patients with congenital hypothyroidism, located in the protein domain of *SLC26A4*. The mutation p.S49R is located in the N-terminal intracellular region, p.D661E and p.H723R are located in the STAS domain of the C-terminal intracellular region of the *SLC26A4*. The remainer 3 mutations were scattered in 12 transmembrane domains of the *SLC26A4*

because TM8 is an ion binding region, mutations in this region may affect the overall ion binding ability, thus affecting gene function. R409H, located in TM10, significantly reduced the membrane localization and iodine transport capacity by 83.7%, a result which is consistent with previous reports. Related studies have shown that this site is an anion-binding site, the mutation directly affect the anion-binding site would have a great impact on gene function. His723 is a conserved site that is located in the STAS domain. The mutation H723R would disrupt the π-cation interaction and polar contact between Tyr530 and His723, again affecting protein function (5). D661E and T485M are located in the STAS domain and TM13, respectively. The two mutation sites have no effect on the localization of the pendrin protein to the cell membrane, but have a significant impact on ion transport, reducing it by about 74% and 67% respectively.

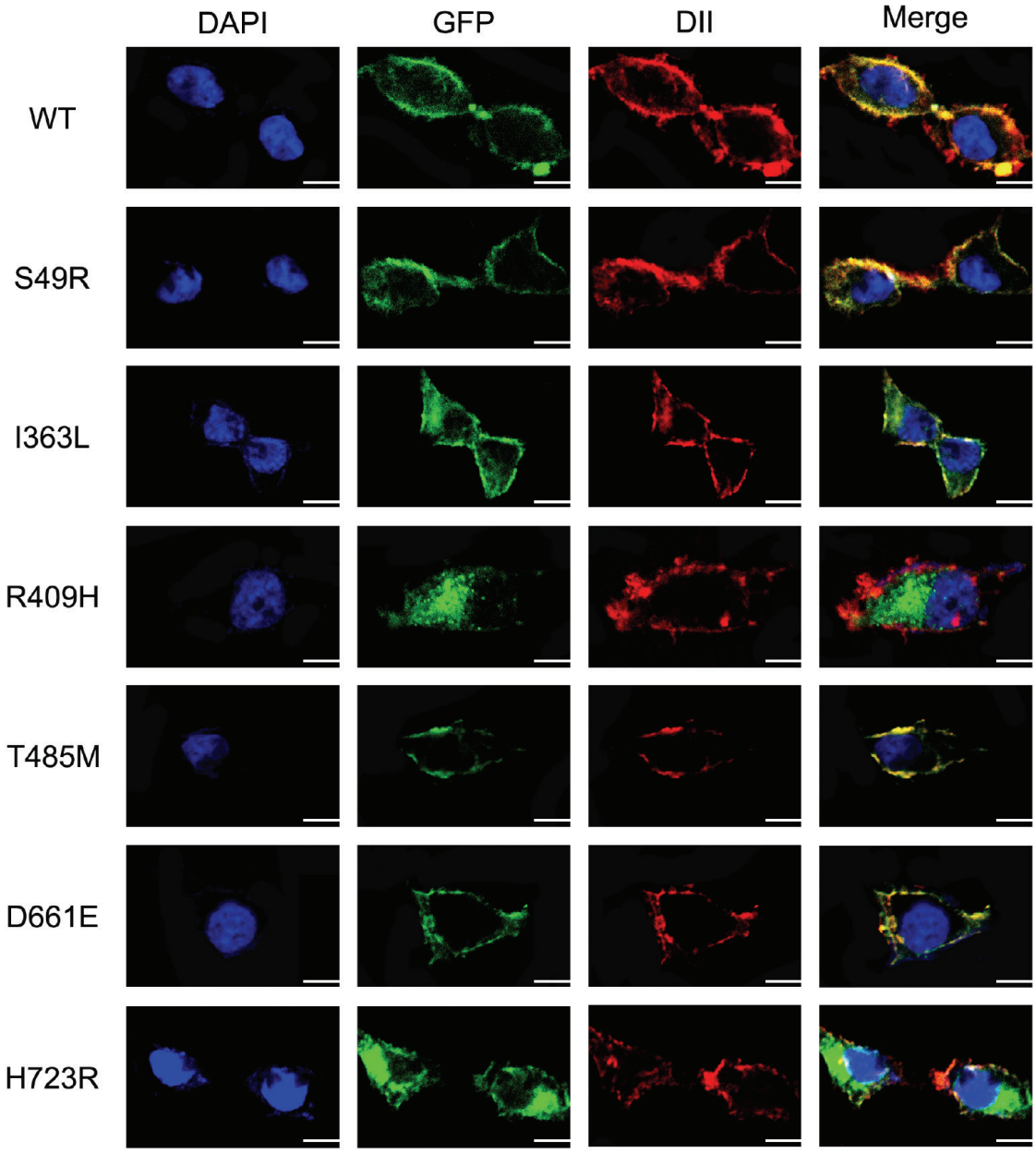

Figure 3. Cellular localization of the six mutants of *SLC26A4* in 293T cells detected by confocal microscopy. Mutant p.S49R, p.T485M, p.D661E of *SLC26A4* show strong membrane fluorescence in 293T cells, which is similar to the WT of *SLC26A4* expressed in the 293T cells, and there was no obvious cytoplasmic retention. Mutants p.I363L, p.R409H, and p.H723R reduce the localization on the cell membrane, and p.R409H, p.H723R show obvious cytoplasmic retention, p.I363L is less. All mutant plasmids were homozygous. Scale: 10 μm

WT: wild-type

There are mutants that have been characterized as having an effect at the cell surface but with reduced function, such as G209V, F335L, M775T. So, the mechanism of the effect of D661E and T485M on the protein may be similar to these loci, which needs further study (5,17,23).

SLC26A4 is expressed in the inner ear and thyroid (17). In the inner ear, *SLC26A4* functions as a Cl^-/HCO_3^- exchanger and regulates the balance of endolymphatic ions, thus affecting the function of the inner ear (24). Homozygous mutations in *SLC26A4* lead to EVA, which is the most common inner ear malformation associated with sensorineural deafness in children (25). However, some patients with EVA carried a heterozygous mutation in *SLC26A4*, rather than a biallelic mutation, suggesting that there are other genetic factors involved in the occurrence of EVA. A study conducted by Yang et al (26) confirmed this hypothesis. They identified heterozygous mutations in *SLC26A4* and KCNJ10 from one patients with EVA. In our study, through follow-up, we found that these 10 patients with CH did not have deafness, but they refused to carry out imaging examination of the inner ear, and thus we were unable to determine whether there was EVA.

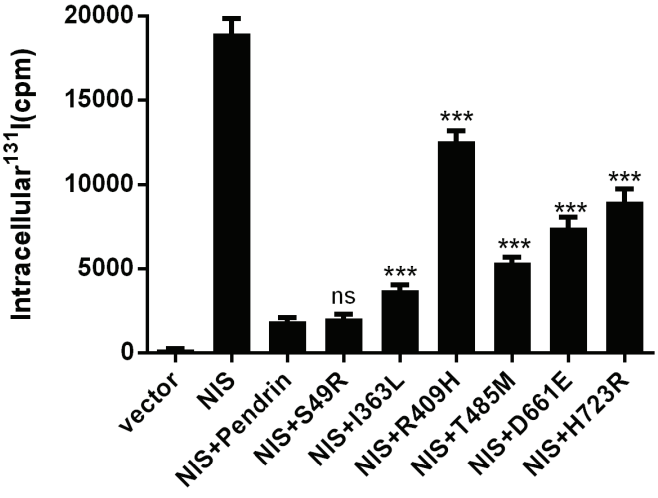

Figure 4. The effect of the mutations in *SLC26A4* on iodine transport capacity in 293T cells. Intracellular iodide accumulation in 293T cells after co-transfection with sodium-iodide symporter (*NIS*) and wild-type (WT) or the mutant *SLC26A4*-pEGFP-N2 plasmids were detected by γ-counter. Intracellular iodide accumulation in 293T cells after co-transfection with *NIS* and mutants palsmids from the p.I363L, p.R409H, p.T485M, p.D661E and p.H723R variants were significantly increased, compared to those co-transfected with NIS and WT (pendrin) *SLC26A4*-pEGFP-N2 plasmid, indicating that these mutants reduced iodide efflux mediated by *SLC26A4*. All mutant plasmids were homozygous. The data are shown as mean ± standard error for three independent experiments. Statistical analysis used Welch's t-test

*ns: No statistical difference, ***p < 0.001*

Pathogenic mutations in *SLC26A4* are well recognized as being the pathogenic mutation in Pendred syndrome (PS), which is an autosomal recessive disorder characterized by sensorineural hearing loss, and goiter and some cases may be identified in the neonatal period with CH (27). However, the thyroid phenotype of PS patients is not clearly defined. In 2014, Ladsous et al (28) found that about 30% of PS patients will present with CH and 78.9% patients have goiter in PS patients with biallelic mutations of *SLC26A4*. The researchers speculated that these differences in PS thyroid phenotype in patients with biallelic mutations of *SLC26A4* might be due to different iodine intakes, as most of the patients with PS presented with hypothyroidism in a moderately iodine deficient region in France, but PS patients from Japan and Korea, regions with high iodine intake, were euthyroid (28,29). Although the thyroid phenotype in human patients with PS seemed to be related to iodine intake, lower iodine intake did not lead to goiter and hypothyroidism in the *SLC26A4* knockout mice (30), indicating that other genetic factors or environmental factors might be involved in the pathogentic mechanism resulting in goiter and hypothyroidism in humans with *SLC26A4* mutation. Indeed, among the 10 patients with *SLC26A4* heterozygous mutation, eight patients carried other mutations in genes associated with CH in our cohort (Table 1). Patient 42 and 259 carried compound heterozygous mutations in the *TG* gene, which is the key gene in the thyroid hormone synthesis and was the pathogenic gene in these two patients. Patient 51 and 247 carried biallelic mutation in DUOX2, which is also involved in thyroid hormone synthesis, but the association between DUOX2 variants and CH is less definite and thus the DUOX2 variants in our patients may be pathogenetic. Four of the remaining six patients carried at least one heterozygous mutation in another candidate gene for CH, while the remaining two patients were found to only have heterozygous mutation in *SLC26A4* amongst the 21 CH-associated genes tested. Although we confirmed that the mutations in *SLC26A4* in our patients with CH could decreased the ability of the iodide transport *in vitro*, none of the parents of these patients, some of whom were also carriers, did not have hypothyroidism, suggesting that these heterozygous mutations in *SLC26A4* are probably not the pathogenic gene for our patients who had all been diagnosed with CH and that there are other genetic or environmental factors which might lead to CH. As EVA could be caused by the heterozygous mutation in *SLC26A4* combining with the heterozygous mutation of KCNJ10, and a previous study has reported that heterozygous mutations in DUOXA2 and DUOX2 might lead to CH in a 4-year-old patient (31), it cannot be excluded that those

patients with monoallelic mutation of *SLC26A4* may combine with other, unidentified, gene variants to cause hypothyroidism.

Study Limitations

The sample size of this study was limited, and no individuals with a homozygous mutation in *SLC26A4* were found. In addition, we could not further elucidate the pathogenesis of *SLC26A4*. Furthermore, the candidate gene panel used in this study did not include SLC26A7, which can also lead to hypothyroidism.

Conclusion

In this study, we identified seven distinct variants of *SLC26A4* in 10 patients from a cohort of 273 Chinese patients with CH. Functional studies showed that five out of six missense mutations in *SLC26A4*, including one novel mutation, p.I363L, have variable effects on protein function. However, because these mutations were all heterozygous mutations, and 8 out of ten patients also carried variants in other CH candidate genes, the pathogenesis of CH in these patients cannot be explained by these *SLC26A4* variants. The pathogenesis of CH in these patients needs further study.

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Ethics

Ethics Committee Approval: The study was approved by the Ethics Committee of Shanghai Ninth People's Hospital affiliated to Shanghai JiaoTong University School of Medicine (decision no: 2016-76-T33, date: 2016-08-03).

Informed Consent: Informed consent was obtained from all patients or their legal guardians, and all unaffected family members who participated in the study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Cao-Xu Zhang, Feng Sun, Wen-Jiao Zhu, Rui-Jia Zhang, Ya Fang, Chen-Yan Yan, Concept: Shuang-Xia Zhao, Huai-Dong Song, Design: Chang-Run Zhang, Data Collection or Processing: Chang-Run Zhang, Qian-Yue Zhang, Ying-Xia Ying, Analysis or Interpretation: Chang-Run Zhang, Yuan-Ping Shi, Literature Search: Chang-Run Zhang, Writing: Chang-Run Zhang, Shuang-Xia Zhao.

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