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

Zhang et al. SLC26A4 Mutation in Congenital Hypothyroidism

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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).

What this study adds?
We identified 7 distinct variants of SLC26A4, including one novel mutation, in Chinese patients with CH. Functional studies showed that five out of six missense mutations have different effects on gene function. It provides 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: 273 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 found and analyzed. The function of 6 missense mutation in SLC26A4 were further investigated in vitro.


Conclusion: Our study indicated that the prevalence of SLC26A4 mutations was 3.66% in the 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, which revealed important role of the Ile363, Arg409, Thr485, Asp661, His723 residues in function of SLC26A4. Because these mutations are heterozygous mutations, the pathogenesis of these patients cannot be explained, and the pathogenesis of these patients needs further study.

Keywords: Congenital hypothyroidism, Next-generation sequencing, SLC26A4, Mutation, Function

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Introduction

Congenital hypothyroidism (CH) is a common neonatal endocrine disorder. Unless treated in the first few months of life, severe congenital hypothyroidism can lead to growth retardation and permanent intellectual disability (1). The incidence of congenital hypothyroidism was about 1:4000 in 1970, and the incidence of the disease increased to 1:2000 in the past decades (2). About 85% cases of CH are caused by abnormal thyroid development (thyroid dysgenesis), but only 2%-5% thyroid dysgenesis case can identify the pathogenic genes, such as thyroid stimulating hormone receptor (TSHR), PAX8, GLIS3, NKX2.1 and FOXE1 (3). The remaining 15% cases of CH are due to defects of thyroid hormone biosynthesis (dyshormonogenesis). Dyshormonogenesis is often caused by mutations in genes that involved in the pathway of thyroid hormone synthesis, such as thyroperoxidase (TPO), dual oxidase 2 (DUOX2), sodium-iodide symporter (NIS; SLC5A5) and apical iodide transporter pendrin (PDS; SLC26A4) (4).

SLC26A4 encodes pendrin, a multi transmembrane protein composed of 780 amino acids, consisting of 12-14 transmembrane segments and a segment of intracellular STAS functional domain(5-9). 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 could lead to CH (11), however, the biallelic mutation of SLC26A4 have not been found in Chinese patients with CH. In order to evaluate the roles of SLC26A4 in the pathogenesis of the Chinese CH patients, our study identified SLC26A4 mutations in the Chinese patients with CH and analyzed the function of the mutation in vitro.

Materials and methods

Clinical subjects

We enrolled 273 CH patients (141 females and 132 males) 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 thyroid stimulating hormone (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, triiodothyronine (T3), thyroxin (T4), free T3 (FT3), and free T4 (FT4) in serum were determined by performing an immune-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): 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, however, after stopping treatment, a rise in TSH and a drop in FT4 were observed again. Apart from that, there are some patients come from outpatient clinics who are using hormone 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 Jiao Tong University School of Medicine(2016-76-T33). Informed consent was obtained from all patients or their legal
guardians, and all unaffected family members who participated to the study.

**Next-generation sequencing (NGS)**

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). 21 previously reported possible causative genes for CH: TPO (GenBank reference sequence: NM_000547), SLC5A5 (NM_000453), 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), THRBN (NM_00125263), DUOX1 (NM_017434), DUOXA1 (NM_001276268), GNAS (NM_016592), SLC16A2 (NM_006517) and HHEX (NM_002729) were selected 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) according to the manufacturer's protocol. The primers were designed using iPLEX Assay Design software (Sequenom). The HiSeq3000 platform (Illumina, San Diego, CA) 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 NGS data and verification using Sanger sequencing**

We analyzed raw sequence data in fastq format and obtained the quality scores by following the method indicated by previous studies (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). We filtered out the variants with frequencies > 1% in the dbSNP 135 and ESP6500 v2 databases and focused on the functional (protein altering) variants (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). The missense mutations including p.S49R, p.I363L, p.R409H, p.T485M, p.D661E and p.H723R were introduced into the SLC26A4-pEGFP-N2 WT plasmid by Fast Mutagenesis System kit (TransGen Biotech) 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) supplemented with 10% fetal bovine serum (FBS; Sigma Aldrich) at 37°C in a humidified atmosphere containing 5% CO2. Transfections were performed on cells by Lipofectamine™ 2000 Transfection Reagent (Invitrogen™) following the manufacturer's instructions. Cells were plated in 20mm 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% parafomaldehyde (PFA) for 30 minutes. After washing with PBS(1X), cells were stained with the membrane probe DiI (Beyotime, Haimen, China) at 37℃ for 5-10 min, then nuclear were stained with DAPI (Beyotime Biotech) 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).

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 1ml serum-free medium containing 3μCi at 5KBq/ml as the only source of iodide. The cells were then washed briefly in HBSS buffer and then incubated with 1ml HBSS for 5 minutes after which HBSS was removed. The cells were solubilized by the addition of 1ml 1N 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.

Results

Clinical characteristics of patients with CH

In our research, 273 patients with CH were enrolled (141 females and 132 males). The median value of serum TSH level and serum FT4 level were 54.075 uIU/mL and 0.718 ng/dL, respectively. All of them have normal hearing. As for whether these children have EVA is unknown because their families refuse to check.

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%. We identified 7 heterozygous mutations of SLC26A4 in 10 CH patients, including one novel mutation (p.I363L). And 8 of these 10 patients also carry the mutations in other candidate gene for CH (Table 1). All mutation sites were verified by Sanger sequencing (Figure 1). But Patient 190 was unable to get to the Sanger sequencing result because of DNA sample damage and patient refused to provide it again. The frequency of SLC26A4 mutation in the Chinese patients with CH was 3.66% (10/273). Among the 7 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 played a key role in the membrane location of SLC26A4. The remaining mutation sites were scattered in 12 transmembrane 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 localization of SLC26A4, we expressed wild-type and mutants of the SLC26A4-pEGFP-N2 plasmid in 293T cells and observed localization using a confocal fluorescence microscope. Although these cells lack the polarization of thyroid follicular cells, wild-type 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 wild-type SLC26A4. Mutants p.R409H and h.H273R can not reach the cell membrane and obviously stay in the cytoplasm. Novel mutant p.I363L showed partly retained in cytoplasm, but most are reached the cell membrane. The remain mutants p.T485M, p.D661E, p.S49R had no effect on cell membrane localization (Figure 3).

Assess the ability of iodide transport for the wild-type and mutants of the SLC26A4

SLC26A4 is speculated to mediate the iodide efflux in 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 wild-type 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 a accumulation of radioiodide (131I) in the cells, however, there were only a small amount of 131I retained in the 293T cells after co-transfected with NIS and
wild-type SLC26A4 plasmids. However, compared to the 293T cells co-transfected with NIS and wild-type SLC26A4 plasmids, except for one mutants (p.S49R), the cells co-transfected with NIS and mutants of SLC26A4 resulted in significantly decreased of iodine efflux, indicating that these mutants lead to decrease their ability of the iodide transport in 293T cells (Figure 4).

Discussion

Congenital hypothyroidism (CH) is a common endocrine system disease with prevalence ranging from 1:2000 to 1:4000 in newborns (19) which is closely related to genetic factors, but most patients did not find the pathogenic gene. Therefore, it is most important to expand the mutation spectrum of the pathogenic genes in patients with CH. In our study, we screened the mutation of SLC26A4 in the Chinese patients with CH and found seven different heterozygous variants in 10 individuals (10/273, 3.66%). The prevalence of SLC26A4 mutations in our study was similar to previous study that screened SLC26A4 mutation in CH patients from Guangxi Zhuang Autonomous Region, China (20), and all the mutations detected were heterozygous mutations, which could not explain the pathogenesis of the patients. These findings suggested that SLC26A4 might be an uncommon pathogenic gene for congenital hypothyroidism in Chinese population.

SLC26A4 is a member of the SLC26 anion transporter family, encodes pendrin which were originally predicted to contain 12 transmembrane (TM) segments (21), however, 14 transmembrane segments were subsequently confirmed by Gorbunov et al (22). These regions contain a lot of anion-binding site or substrate-binding site, such as TM1, TM3, TM10, which would affect the function of this gene (5). The protein also has an STAS domain in the cytosol, which are central to 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 7 mutation sites of SLC26A4 have been identified, including a new one that has not been reported. S49R located in the N-terminal intracellular segment, our functional experiments confirmed that the mutation did not affect membrane localization and ion transport. It may be that the site is not an ion binding site, so the mutation has little effect on gene function. New mutation site I363L located in the TM8 which was an anion-binding site (5). Our study confirmed that mutation I363L affects the membrane localization of SLC26A4 slightly and reduce its ability to transport iodine ions (about 53%). We speculate that 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 the TM10, consistent with other research results, this mutation significantly reduced the membrane localization and iodine transport capacity (about 83.7%). 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 located in STAS domain, mutation of H723R would disrupt the π-cation interaction and polar contact between Tyr530 and His723, thus affecting gene function (5). D661E and T485M are located in the STAS domain and TM13, respectively. The two mutation sites have no effect on the location of cell membrane, but have great influence on ion transport (about 74%, 67% respectively). There are mutants that have been characterized at the cell surface but with reduced function, such as G209V, F335L, M775T. So, the mechanism of the effect of D661E and T485M on genes may be similar to these loci, which needs further study (5; 17; 23).

SLC26A4 is expressed in inner ear and thyroid (17). In inner ear, SLC26A4 as a Cl-/HCO3- exchanger, regulates the balance of endolympathic ions, thus affecting the function of the inner ear (24). It is reported that the homozygous mutation in SLC26A4 lead to enlarged vestibular aqueduct (EVA), which is the most common inner ear malformation associated with sensorineural deafness in children (25). However, some patients with EVA carried the heterozygous mutation in SLC26A4, rather than biallelic mutation in SLC26A4, indicating that there are other genetic factors involved in the occurrence of EVA. Another study conducted by Tao Yang et al had confirmed this hypothesis, they identified heterozygous mutations in SLC26A4 and KCNJ10 from one patients with EVA (26). 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 inner ear, unable to determine whether there was EVA.

The deficiency of SLC26A4 was well known as the pathogenic gene for Pendred syndrome (PS), which was an autosomal recessive disorder characterized by sensorineural hearing loss, goiter, and some case may have CH (27). However, the thyroid phenotype of PS patients has not been determined clearly. In 2014, Ladsous found that about 30% patients will present CH and 78.9% patients have goiter in PS patients who were identified carried biallelic mutations in SLC26A4 (28). The researchers speculated that the difference of the thyroid phenotype in patients with biallelic mutations of SLC26A4 might be due to the different iodine intakes, such as the most of the patients with PS should be presented hypothyroidism in a moderately iodine deficient region in France, however, PS patients in Japan and Korea, the regions with high iodine intakes, were euthyroid (28; 29). Although the thyroid phenotypes in human patients with PS seemed to be related to iodine intakes, the lower iodine intakes 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 formation of goiter and hypothyroidism in the patients with SLC26A4 mutation. Indeed, among the 10 patients with SLC26A4 heterozygous mutation, 8 patients carried other gene mutations in our study (Table 1). Patient 42 and 259 carried compound heterozygous mutation of thyroglobulin (TG), which is the key gene in the thyroid hormone synthesis and were responsible for the pathogenesis of the two patients. Patient 51 and 247 carried biallelic mutation in dual oxidase 2 (DUOX2), which is involved in thyroid hormone synthesis and might be the pathogenesis of the two patients. Four of the remaining six patients carried at least one heterozygous mutation in other candidate genes for congenital hypothyroidism, and two patients only carried heterozygous mutation in SLC26A4. Although we confirmed that the mutations in SLC26A4 from our patients with CH could decreased the ability of the iodide transport in vitro, one of the parents of these patients, who are also carriers, do not show hypothyroidism, indicating that heterozygous mutation in SLC26A4 might not be pathogenic gene for the patients with CH and there are other genetic or environmental...
factors might be lead to CH. Because EVA could be caused by the heterozygous mutation in SLC26A4 combining with the heterozygous mutation of KCNJ10, and previous study has reported that heterozygous mutations in DUOX2 and DUOX2 might lead to CH in a 4-year-old patient (31), thus, it is not excluded that those patients with monoallelic mutation of SLC26A4 may combine with other gene to cause hypothyroidism.

**Study Limitations**

We evaluated the mutation rate of SLC26A4 in Chinese hypothyroid population, indicating that SLC26A4 is not a common pathogenic gene, and the functional experiment also provides an important basis for the follow-up research. Unfortunately, the sample size of this study was limited, and no individuals with a homozygous mutation of SLC26A4 were found, and could not further elucidate the pathogenesis of SLC26A4. Apart from that, it is pity that the candidate gene mentioned in this study doesn’t include SLC26A7 which can lead to hypothyroidism.

**Conclusion**

In this study, we identified 7 distinct variants of SLC26A4 in 10 patients from 273 Chinese patients with CH. Functional studies showed that five out of 6 missense mutations in SLC26A4, including one novel mutation p.I363L, have different effects on gene function. However, because these mutations are heterozygous mutations, the pathogenesis of these patients can not be explained, and the pathogenesis of these patients needs further study.

**Acknowledgments**

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**Author disclosure statement**

The authors declare no competing financial interest.

**CRediT authorship contribution statement**

Chang-Run Zhang: Writing-original draft, Data curation, Methodology. Yuan-Ping Shi: Methodology. Cao-Xu Zhang: Supervision. Feng Sun, Rui-Jia Zhang, Ya Fang: Resources. Chen-Yan Yan, Wen-Jiao Zhu: Project administration. Qian-Yue Zhang, Ying-Xia Ying: Validation. Shuang-Xia Zhao: Funding acquisition, Formal analysis, Writing-review & editing. Huai-Dong Song: Funding acquisition, Methodology, Conceptualization, Investigation.

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Figure 1.
Figure 2.

extracellular

Figure 3.

intracellular
Figure 4.
Table 1. The clinical data and genetic characteristics of the 10 CH patients with mutation of SLC26A4

<table>
<thead>
<tr>
<th>ID</th>
<th>Gender</th>
<th>Age (day)</th>
<th>Thyroid ultrasound</th>
<th>FT4 (0.58 – 1.64) ng/dL</th>
<th>TSH (0.34 – 5.6) uIU/mL</th>
<th>Mutation information</th>
<th>The frequency observed in public databases</th>
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</tbody>
</table>

Abbreviations: FT4, free thyroxine; TSH, thyroid-stimulating hormone; NA, not available.