Clinica Chimica Acta 497 (2019) 147–152

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/cca

Mutation screening of the *TSHR* gene in 220 Chinese patients with congenital hypothyroidism

Ya Fang[a,1], Feng Sun[a,1], Rui-Jia Zhang[a], Chang-Run Zhang[a], Chen-Yan Yan[a], Zheng Zhou[a], Qian-Yue Zhang[a], Lu Li[a], Ying-Xia Ying[a], Shuang-Xia Zhao[a], Jun Liang[b,2], Huai-Dong Song[a,b,*]

[a] The Core Laboratory in Medical Center of Clinical Research, Department of Endocrinology, Shanghai Ninth People's Hospital, State Key Laboratory of Medical Genomics, Shanghai Jiao Tong University (SJTU), School of Medicine, Shanghai 200011, China
[b] Department of Endocrinology, The Central Hospital of Xuzhou Affiliated to Xuzhou Medical College, Xuzhou, Jiangsu Province 221109, China

ARTICLE INFO

Keywords:
Congenital hypothyroidism
Next-generation sequencing
TSHR
Mutation

ABSTRACT

Background: Defects in the human thyroid stimulating hormone receptor (*TSHR*) gene are reported to be one of the causes of congenital hypothyroidism (CH). We aimed to identify mutations in Chinese patients with CH and analyze the relationships between *TSHR* phenotypes and clinical phenotypes.

Methods: 220 patients with primary CH were screened for *TSHR* mutations by performing next-generation sequencing. All the exons and exon–intron boundaries of *TSHR* were analyzed. The function of 8 mutants in *TSHR* were further investigated *in vitro*.

Results: Among 220 patients with CH, 15 distinct TSHR mutations were identified in 13 patients (5.91%, 13/220, including our previous reported 110 patients, carried with 10 mutations in 8 patients). We found five distinct mutations in the additional cohort of 110 CH patients and identified 7 mutations (including a novel mutation, p.S567R) were loss-of-function mutations.

Conclusion: Our study indicated that the prevalence of *TSHR* mutations was 5.91% among studied Chinese patients with CH. One novel TSHR variant was found and four genetic alterations revealed important role of the Ile216, Ala275, Asn372, Ser567 residues in signaling.

1. Introduction

Congenital hypothyroidism (CH) is a common neonatal endocrine disorder, and characterized by impaired neurodevelopment as well as physical growth and development. The prevalence rate of CH among neonates is 1:3000–1:4500 and 1:2000 in the Caucasian [1] and Chinese populations [2], respectively. Several reports indicated that various genetic factors play important roles in the CH pathogenesis. Previous studies report that CH is associated with > 20 genes [3–6], including thyroid stimulating hormone receptor (*TSHR*), which is one of the candidate genes that is widely investigated with respect to its variants [7,8].

To a certain extent, thyroid growth and the synthesis of thyroid hormones depend on the levels of TSH and TSHR. The protein, encoded by *TSHR*, is a G-protein-coupled receptor that consists of seven transmembrane-spanning regions and a large extracellular domain bridged by a hinge region. After TSH binds and activates TSHR, both the Gs/cAMP and Gq/11 pathways play an important role in the subsequent signal transduction. These two pathways are involved in thyroglobulin iodination and cell proliferation while Gs pathway is also responsible for iodine uptake regulation of the thyrocytes [9,10]. The gain-of-function mutations of *TSHR* are associated with hyperfunction thyroid adenomas and non-autoimmune hyperthyroidism [11]. However, the loss-of-function (LOF) mutations of *TSHR* lead to TSH resistance, which causes CH [12]. *TSHR* mutations contribute to various LOF phenotypes *via* multiple molecular mechanisms. Different mutation positions in *TSHR* cause impairment through distinct pathways, for example, the location of receptors on the cell surface, ligand binding ability of the receptor, and corresponding signal transduction are affected [13].

In our previous study, we had screened 21 candidate genes of congenital hypothyroidism in a cohort of 110 Chinese patients with primary CH, and we found 8 patients carried *TSHR* mutations [14]. In

* Corresponding author at: Endocrinology and Molecular Biology, The Core Laboratory in Medical Center of Clinical Research, Department of Endocrinology, Shanghai Ninth People's Hospital, State Key Laboratory of Medical Genomics, Shanghai Jiao Tong University (SJTU), School of Medicine, Shanghai 200011, China.
E-mail address: huaidong_s1966@163.com (H.-D. Song).
[1] The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint first authors.
[2] The authors wish it to be known that, in their opinion, the last two authors should be regarded as joint corresponding authors.

https://doi.org/10.1016/j.cca.2019.07.031
Received 13 May 2019; Received in revised form 15 July 2019; Accepted 26 July 2019
Available online 26 July 2019
0009-8981/

our present study, we expanded samples for screening the *TSHR* mutations in patients with CH and characterized the phenotypes of patients with CH who carried *TSHR* mutations. We further evaluated the functions of wild type (WT) TSHR and 8 mutants in human embryonic kidney 293 cell line that contains the SV40 T-antigen (293 T cells).

2. Materials and methods

2.1. Clinical subjects

In the Chinese population, neonates with CH were screened based on the TSH determination of TSH level using filter-paper blood spots (obtained through heel prick) acquired during 3–5 days after birth. The diagnostic criteria to establish permanent CH in patients was referred from our previous study [14]. In brief, in infants with elevated TSH levels (≥10 mU/L) during the initial screening, 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 (UniCelDxI 800, Beckman, USA). The diagnosis of permanent CH was confirmed in infants based on the following criteria: (i) elevated TSH levels, (ii) T4 or FT4 levels lower than the reference range, and (iii) restoration of normal thyroid parameters during receiving replacement therapy with L-thyroxine, but recurrent elevated TSH level and reduced FT4 level after the termination of treatment. In this study, we included unrelated infants with CH (n = 220) (Table 1). A written consent was obtained from their parents, and the study was approved by the Ethics Committee of Shanghai Ninth People's Hospital affiliated to Shanghai Jiao Tong University School of Medicine.

2.2. 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 [15]. All the exons and exon–intron boundaries of *TSHR* (GenBank reference sequence: NM_003235) 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 [16].

2.3. Calling of TSHR 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 [16,17]. 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 [14]. We screened the variants with frequencies > 1% in the dbSNP 135 and ESP6500 v2 databases to pass single nucleotide polymorphisms and focused on the identification of functional variants (that affect the protein function) in duplicate samples. The selected variants were validated by performing Sanger sequencing.

2.4. Construction of the TSHR mutants through site-directed mutagenesis

Human WT cDNA that encodes *TSHR* was cloned into p-enhanced green fluorescent protein (EGFP)-N2 plasmid (TransGen Biotech). The missense mutations including p.G132R, p.I216T, p.A275T, p.N372 T, p.R450H, p.R528S, p.S567R and p.S716G were introduced into the TSHR-pEGFP-N2WT plasmid using Fast Mutagenesis System kit (TransGen Biotech) by following the manufacturer's protocol. All the final plasmid constructs were validated by performing Sanger sequencing.

2.5. Gs/cAMP signaling assay

293 T cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/high-glucose medium (Gibco) supplemented with 10% fetal bovine serum (FBS; Sigma Aldrich). The WT and mutant TSHR-pEGFP-N2 plasmids were transiently transfected into 293 T cells using lipofectamine 2000 kit (Life Technologies) following the manufacturer's instructions. 0.5×10^5 293 T cells / well were plated in 24-well plates (Corning). After 24 h incubation, these cells were transfected with plasmid DNA (0.5 μg, pEGFP-N2 that contains either the WT or mutant TSHR or empty pEGFP-N2), cultured in opti-MEM medium without FBS for 4–6 h, and then the spent medium was replaced with fresh DMEM with 10% FBS. The cAMP concentration in transfected cells was measured using the cAMP assay kit (R&D Systems assay, USA) [18]. Briefly, after transfection and incubation for 48 h, medium was removed and cells were incubated in Krebs-Ringer phosphate buffer for 15 min at 37 °C. Then, 0, 1, 10, or 100 IU/L bovine TSH (bTSH; Sigma Aldrich) was added into the aforementioned buffer, and cells were incubated for another 1 h at 37 °C. Cell lysates were prepared and cAMP concentration in these transfected cells was measured by following the manufacturer's instructions of the cAMP assay kit.

2.6. Gq/11 signaling assay

Gq/11 signaling of TSHR variants were indirectly examined through the firefly luciferase activity of 293 T cells which is regulated by the nuclear factor of activated T-cells response element (NFATRE) promoter of the reporter plasmid co-transfected with TSHR-containing constructs. 1×10^4 /well 293 T cells were seeded onto 96-well plates (Corning). The following day, the cells were transfected with total 100 ng plasmid DNA/well (20 ng pEGFP-N2 containing WT or mutant TSHR-s or empty pEGFP-N2, 8 ng renilla luciferase reporter plasmid-Promega, and 72 ng firefly luciferase reporter plasmid-Promega). After transfection for 48 h, the cells were incubated in DMEM/high-glucose medium with or without 100 IU/L bTSH for 6 h at 37 °C. Firefly and renillla luminescence intensities of the cells were examined in black flat bottom 96-well plates following the protocol of the Dual-Luciferase® Reporter Assay System.

Table 1
Clinical characteristics of 184 patients with congenital hypothyroidism.

	All patients (n = 184)	Male patients (n = 110)	Female patients (n = 110)	Reference range
Age at diagnosis, days	28.701 ± 16.869	29.237. ± 16.039	28.623 ± 18.117	
FT3,pg/mL	2.90 ± 1.227	2.99 ± 1.188	2.79 ± 1.249	2.5–3.9
FT4,ng/dL	0.612 ± 0.371	0.658 ± 0.434	0.574 ± 0.306	0.58–1.64
TSH, uIU/mL	78.978 ± 49.50	73.166 ± 43.753	84.797 ± 55.361	0.34–5.6
dose of T4(ug/day)	42.639 ± 30.063	41.41 ± 30.396	45.216 ± 30.344	

Abbreviations: FT3, free triiodothyronine; FT4, free thyroxine; TSH, thyroid-stimulating hormone. Results are expressed as mean ± SD.

2.7. Statistical analysis

Statistical analysis results were presented as mean ± SEM and carried out using SPSS 19.0. Welch's *t*-test was used to compare the activities of cAMP and Gq/11 signaling. Mann-Whitney *U* test to compare the serum TSH and FT4 levels among two groups. $P < .05$ was considered as statistically significant value.

3. Results

3.1. Clinical characteristics of patients with CH

In our research, 220 patients with CH were enrolled, among whom 110 were male. The median value of age at diagnosis, serum TSH level, and serum FT4 level, were 28.7 days, 78.98 uIU/mL, and 0.612 ng/dL, respectively. In our cohort, the average maintenance dose of thyroid hormones (T4) was 42.639 μg/day. These features are shown in Table1.

3.2. Screening the missense mutations of TSHR in Chinese patients with CH

All the exons and exon-intron boundaries were amplified by performing multiplex PCR using customized primers designed to generate 200–250-bp amplicons. After the quality control assessment, the average region of *TSHR* screened by performing sequencing up to ≥20 × depth was 97.67%. In our previous study, among 110 Chinese CH patients, we found 8 patients carried 10 distinct *TSHR* mutations, including one hot mutation (p.R450H). And three patients carried the biallelic mutations of *TSHR* (p.I216T + p.A275T, p.G132R + p.R450H and p.R450H + p.R528S). In the present study, among the additional cohort of 110 Chinese patients with CH, we identified 5 mutations of *TSHR* in 5 patients (none had biallelic mutations), including one novel mutation (p.S567R) (Table 2). The frequency of TSHR mutation was 5.91% (13/220). These mutations were confirmed by performing Sanger sequencing.

3.3. Effect of mutant receptors on Gs/cAMP and Gq/11 signaling pathway

TSHR consists of an extracellular N-terminal domain (encoded by the first 9 exons and a part of exon 10), intracellular domain, and 7 membrane-spanning domains (these two domains are encoded by exon 10). Our study indicated that most of the *TSHR* mutations are located in exon 10 (Fig. 1). The 5 mutations (p.G132R, p.I216T, p.A275T, p.R450H and p.R528S) detected in 3 patients with biallelic mutations and 3 mutations (p.N372 T, p.S567R and p.S716G) that had never been identified were selected to assess their molecular functions by examining their signaling properties in *in vitro* experiments. The potential of mutant TSHRs to responds to bTSH was detected in 293 T cells transfected with mutant TSHRs by evaluating the cAMP production and Gq/11 signaling. Compared to 293 T cells transfected with WT-TSHR, the cAMP production in response to bTSH was abolished in cells transfected with p.S567R-TSHR mutant and moderately reduced in cells transfected with p.G132R, p.I216T, p.A275T and p.N372 T-TSHR mutants (Fig. 2A). Additionally, p.R450H-TSHR mutant exhibited a mild effect but significantly attenuated cAMP production in response to bTSH (Fig. 2A). However, the p.R528S and p.S716G-TSHR mutants did not affect the cAMP production in response to bTSH in 293 T cells (Fig. 2A). Interestingly, p.G132R, p.I216T, p.A275T, p.N372 T and p.R528S-TSHR mutants moderately affected Gq/11 signaling, while p.R450H and p.S567R-TSHR are characterized by profound loss of Gq/11 signaling (Fig. 2B). p.S716G-TSHR mutants also had no effect on Gq/11 signaling (Fig. 2B).

3.4. Clinical features of patients with CH who carried TSHR mutations

The frequency of *TSHR* mutation carriers in this cohort of Chinese patients with CH was 5.91% (13/220). Among them, 1.36% (3/220), and 4.55% (10/220) were biallelic and monoallelic *TSHR* mutation carriers, respectively (Table 2). Compared with CH patients who carried biallelic *DUOX2* mutations, the TSH levels were lower in CH patients who carried *TSHR* mutations with or without monoallelic *DUOX2* mutations (37.059 ± 31.145 *vs* 90.739 ± 51.684, $P = .006 < 0.05$; Fig. 3). Among the 13 patients with CH who carried *TSHR* mutations, the thyroid sizes information was available for 9 patients, that was detected by performing the ultrasound imaging of thyroid gland. The results indicated that 3 patients exhibited goiter, while 1 patient exhibited thyreoaplasia, and this patient carried a mutation in NK2

Table 2
The clinical and mutation information of 13 mutated CHT patients in our study.

ID	Gender	At diagnose					Mutation information	
		Age (day)	Thyroid ultrasound	FT3 (2.5–3.9) pg/mL	FT4 (0.58–1.64) ng/dL	TSH (0.34–5.6) uIU/mL	*TSHR* mutation	*DUOX2* mutation
1	Female	30	Goiter	2.72	0.41	>100	c.G1576A (p.A526T)	p.R683L, p.D932N, p.R974H, p.L1343F
2	Male	30	NA	NA	NA	NA	c.T647C (p.I216T) c.G823A (p.A275T)	p.S363G
3	Male	NA	Normal	NA	NA	9.398	c.G326A (p.R109Q)	p.R1211H
4	Female	30	Normal	2.19	0.28	>100	c.A1115C (p.N372 T)	p.P122T, p.R683L, p.L1343F
5	Male	45	Goiter	2.38	0.52	19.065	c.G394C (p.G132R) c.G1349A (p.R450H)	No
6	Male	30	Goiter	2.46	0.7	38.583	c.A2146G (p.S716G)	p.K530X, p.R885L
7	Female	30	Thyroaplasia	3.61	1.01	53.51	c.A2252G (p.K751R)	p.N476K
8	Male	30	NA	NA	NA	NA	c.G1349A (p.R450H) c.C1582A (p.R528S)	p.R1211H
9	Male	NA	Normal	4.26	1.09	9.16	c.T1701G (p.S567R)	No
10	Male	NA	NA	NA	NA	9.43	c.T915A (p.S305R)	p.D1260N, p.R432H, p.N232K
11	Male	15	NA	NA	NA	52.5	c.G1060A (p.V354I)	p.R885L
12	Male	31	Normal	4.407	0.55	>100	c.C611T (p.A204V)	p.G1513R
13	Male	33	Normal	3.54	1.14	15.751	c.T491A (p.M164K)	p.R885Q

The thyroid hormone levels at screening of 2 patients were absolute absent, while their parents nuncupated their children had CHT by neonatal.

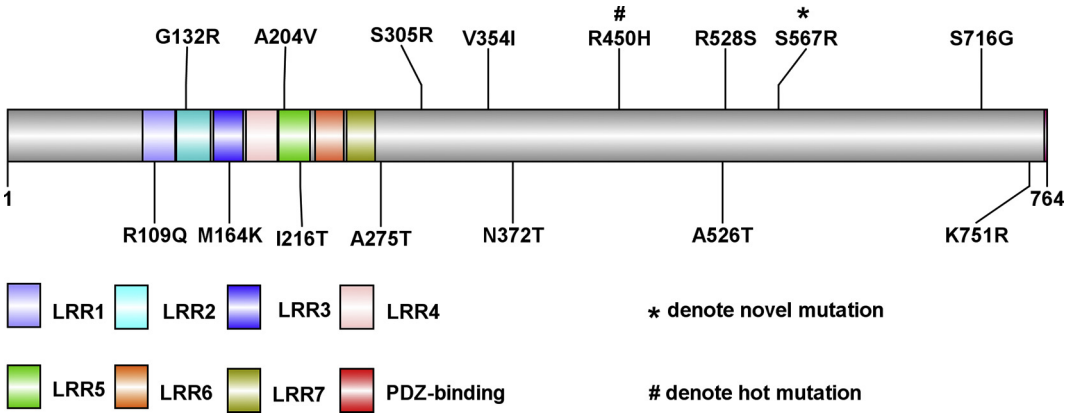

Fig. 1. Mutations in TSHR.
Among the 15 mutations located in TSHR, 6 and 9 were in the initial 9 exons and exon 10, respectively. * and # denote the novel and recurrent mutations, respectively.

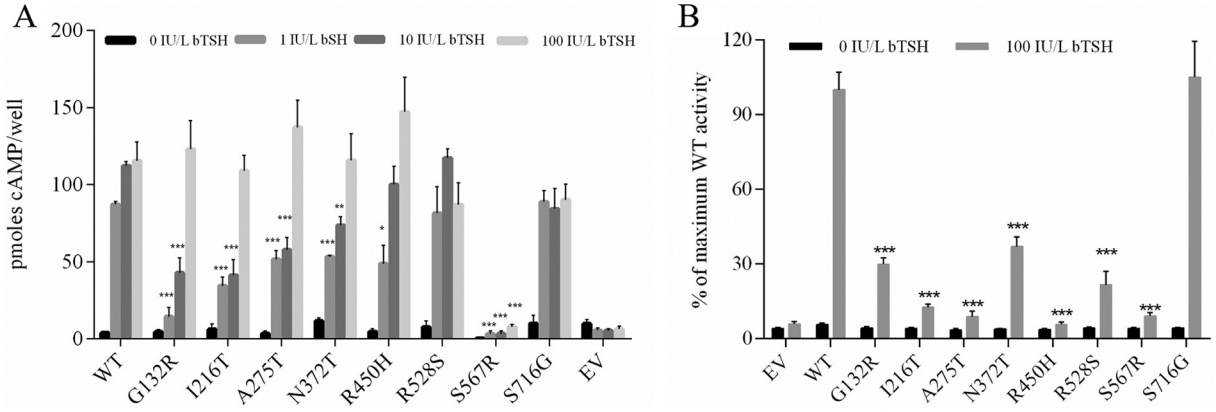

Fig. 2. Signaling properties of the mutant TSHR-s.
A, cAMP signaling pathway in 293 T cells with mutant TSHR-s. After transient transfection for 48 h, the cAMP levels were estimated using various concentrations of bTSH as stimulants (0, 1, 10, or 100 IU/L). The levels are represented as the concentration of intracellular cAMP in 293 T cells. B, Gq/11 signaling of the TSHR variants. 293 T cells were transfected with 100 ng total plasmid per well (20 ng empty vector, WT or mutant TSHR-s vector, 72 ng NFATRE promoter plasmid, 8 ng renilla luciferase reporter plasmid). After transfection for 48 h, cells were incubated with or without 100 U/L of bovine TSH in DMEM medium for 6 h at 37 °C. Then, we measured luciferase activity. The bar diagrams are representative of 3 independent experiments using 3 to 6 individual samples and show mean ± standard error of mean. EV (empty vector) represents intracellular cAMP level and relative NFATRE-luciferase activity in 293 T cells transfected with pEGFP-N2.We used Welch's *t*-test *vs.* wildtype.* denotes $p < .05$, **denotes $p < .01$, ***denotes $p < .001$.

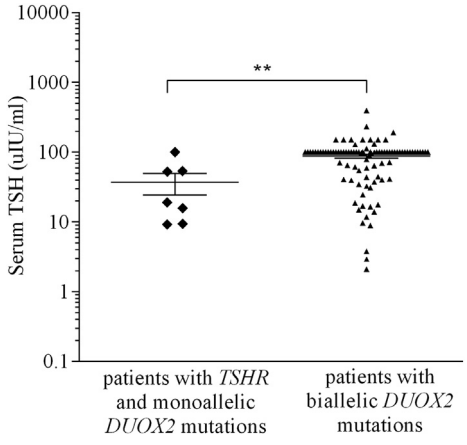

Fig. 3. Distribution of serum TSH.
The serum TSH levels in the patients with CH who carried *TSHR* mutations with or without *DUOX2* biallelic mutations (diamond) and patients who carried biallelic *DUOX2* mutations (triangle). We used Mann-Whitney *U* test to compare the serum TSH levels among two groups, ** denotes $P < .01$.

homeobox 1 *(NKX2–1)* which was identified as a key transcription factor during the thyroid development in our previous study [14] (Table 2).

3.5. Genotype-phenotype correlation analysis

We had identified variants in *DUOX2* previously in our cohort of 220 Chinese CH patients (data of the additional 110 patients were not published in public now). In our 220 cohort patients study, 13 out of 15 patients with *TSHR* mutations carried one or more *DUOX2* mutations. Interestingly, distinct mutations in different subjects may have diverse clinical symptoms.

Patient 5 (P5), was a male patient with p.G132R/p. R450H mutations in *TSHR*. There was no family history of thyroid disease, he was diagnosed at 45 days of age, by which time his serum TSH level was high (19.065uIU/mL) and FT4 level (0.52 ng/dL) was low. and levothyroxine (L-T4) replacement therapy was started immediately. Now, P5 is 7 years old with normal mental development, his height is 132 cm and weight is 26 kg (normal physical development). He takes L-T4 100 μg per day to maintain normal thyroid function.

Patient 1 (P1), was a female patient with p.A526T mutation in *TSHR* and p.R683L, p.D932N, p.R974H, p.L1343F mutations in *DUOX2*. There

was no family history of thyroid disease, she was diagnosed at 30 days of age, by which time his serum TSH level was high (>100uIU/mL) and FT4 level (0.41 ng/dL) was low, and levothyroxine (L-T4) replacement therapy was started immediately. Now, P1 is 6.5 years old with normal mental and physical development). She takes L-T4 75 μg per day to maintain normal thyroid function.

Patient 12 (P12), was a male patient with p.A204V/p.G1513R in *TSHR/DUOX2*. High TSH level (21.3 mU/L) were detected at 7 days of age during neonatal screening. The patient was recalled for further evaluation at 30 days of age, by which time his TSH level > 100 uIU/mL and FT4 level (0.55 ng/dL) was low. His initial dose of L-T4 is 37.5 μg per day. Now he is 2 years old and still takes this dose. His brother also has congenital hypothyroidism, and carried with p.A204V/p.G1513R in *TSHR/DUOX2*. His mother harbored p.A204V in *TSHR* and his father harbored p.G1513R in *DUOX2*, both of them has normal thyroid function.

4. Discussion

In this study, we found that mutations of *TSHR* are common genetic causes of CH in the Chinese population (5.91%, 13/220), as the prevalence of biallelic and monoallelic *TSHR* mutations is 1.36% (3/220), and 4.55% (10/220), respectively. The reported incidence of primary CH is 1:2000 in China [2]. Accordingly, the calculated prevalence of biallelic and monoallelic *TSHR* mutation carriers is 1:1,23,000 and 1:175, respectively, in the Chinese population, assuming the Hardy-Weinberg distribution. Our results are similar to the previous reports by Lábadi et al. [18]. In this cohort of patients with CH (n = 220), among the 13 patients with *TSHR* mutations, 3 carried biallelic *TSHR* mutations. Considering the results of functional experiment, the hypothesis regarding the onset CH owing to *TSHR* mutations could be explained in these 3 patients (patient-2, −5 and − 8). Interestingly, in patients with CH, we found that the TSH levels were lower in 7 *TSHR* mutations carriers with or without monoallelic *DUOX2* mutations than that in biallelic *DUOX2* mutations carriers. This can be explained that most of our patients carried monoallelic *TSHR* mutations, which just lead to partial TSH resistance. P5 carried compound heterozygous mutations in *TSHR*, and he (100 μg/d) needs higher maintenance dose of L-T4 than P1 (75 μg/d, carried with monoallelic *TSHR* mutation and four allelic *DUOX2* mutations) and P12 (37.5 μg/d, carried with one mutation in *TSHR* and *DUOX2*, respectively). Compound heterozygous *THSR* mutations causing severe resistance to TSH is one of possible reasons.

The pathogenesis of patients with CH was categorized into 2 groups, namely, thyroid agenesis and thyroid dyshormonogenesis that account for 80–85% and 15%–20% cases, respectively. In the thyroid gland, agenesis is associated with aberrations in *TSHR*, thyroid transcription factor 1 and 2 (*TTF*-1 *and* -2), and paired-box gene 8 (*PAX*8), while dyshormonogenesisis related to sodium iodide symporter (*NIS*), *DUOX*2, and sodium-independent transporter of chloride and iodide (*SLC*26*A*4) aberrations [19–22]. In this study, among the 9 patients with CH who carried *TSHR* mutations, in whom the thyroid size was measured by performing ultrasound imaging, only a single patient exhibited thyroid hypoplasia (patient-7 with K751R *TSHR* mutant). Moreover, patient-7 carried a mutation in *NKX*2–1 which encodes a transcription factor that affects thyroid development. Notably, among the 3 patients with CH who exhibited goiter, patient-5 carried a biallelic LOF mutation in *TSHR*. These findings indicated that the *TSHR* mutation may associated with thyroid dyshormonogenesis.

TSHR is a G-protein-coupled receptor that consists of seven transmembrane-spanning domains, an intracellular domain, and a large extracellular domain. In *TSHR,* the initial 9 exons and a part of exon 10 encode extracellular domain (containing leucine-rich repeats and hinge region) and the exon 10 encodes all the transmembrane-spanning regions as well as intracellular domain. In our study, we found 15 distinct *TSHR* mutations (including previous reported 10 mutations), among which 9 were located at the exon 10, and these results were consistent with a previous report [13]. Three mutations (p.N372 T, p.S567R, and p.S716G) were located at the hinge region, transmembrane-spanning region, and intracellular domain of *TSHR*, respectively. A previous study indicated that the binding ability of TSHR to bTSH is regulated by leucine-rich repeats [23]. However, the mechanism of TSHR binding ability is not completely known owing to lack of information regarding the hinge region structures. Mueller et al. analyzed 41 single mutants of the hinge region and identified mutations in the F381 to D386 region of TSHR which is a novel TSH binding domain [24]. However, in their study, they did not include the mutation at residue N372, that was found to affect the function of TSHR through both Gs and Gq/11 signaling in our present research. The functional characteristics of p.S567R mutant *TSHR* remained unknown in previous studies. In our study, we demonstrated that the aforementioned mutation causes profound impairment in the Gs and Gq/11 pathway. However, further investigation is essential to understand the mechanisms involved in these manifestations. We found that p.S716G neither interfere with the Gs nor the Gq/11 pathway. Variant p.R450H, is a hotspot mutation in *TSHR* that caused a mild reduction in the cAMP production in response to bTSH compared to WT-TSHR while caused a profound impairment in Gq/11 pathway, which were consistent with previous report [25,26].

There were some limitations in our study. First, although three patients carried with biallelic LOF TSHR mutations, we cannot identify their inherited characteristics for missing clinical and genetic information of these three probands' pedigrees. Second, only a small number of patients carried mutations, so we cannot exactly determine the relationship between genotype and phenotype.

In conclusion, in this study, we reported 13 patients with 15 distinct mutations on TSHR (including our previous reported 10 mutations in 8 patients) and identified function of 8 mutant-TSHR proteins (6 were never uncharacterized before, including one novel mutation). Notably, 3 uncharacterized mutations (p.I216T, p.A275T and p.N372 T) and one novel mutations (p.S567R) impaired the function of TSHR through both Gs/cAMP and Gq/11 pathway during the *in vitro* study.

Funding

This work was partially supported by Chinese National Key Research Program (2017YFC1001801) and the National Natural Science Foundation of China (81430019, 81770786, 81661168016, 31571296, 31501015).

Author disclosure statement

The authors declare that they have no competing interests.

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