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TSHR Variant Screening and Phenotype Analysis in 367 Chinese Patients With Congenital Hypothyroidism

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Background: Genetic defects in the human thyroid-stimulating hormone (TSH) receptor (*TSHR*) gene can cause congenital hypothyroidism (CH). However, the biological functions and comprehensive genotype–phenotype relationships for most *TSHR* variants associated with CH remain unexplored. We aimed to identify *TSHR* variants in Chinese patients with CH, analyze the functions of the variants, and explore the relationships between *TSHR* genotypes and clinical phenotypes.

Methods: In total, 367 patients with CH were recruited for *TSHR* variant screening using whole-exome sequencing. The effects of the variants were evaluated by *in-silico* programs such as SIFT and polyphen2. Furthermore, these variants were transfected into 293T cells to detect their Gs/cyclic AMP and Gq/11 signaling activity.

Results: Among the 367 patients with CH, 17 *TSHR* variants, including three novel variants, were identified in 45 patients, and 18 patients carried biallelic *TSHR* variants. *In vitro* experiments showed that 10 variants were associated with Gs/cyclic AMP and Gq/11 signaling pathway impairment to varying degrees. Patients with *TSHR* biallelic variants had lower serum TSH levels and higher free triiodothyronine and thyroxine levels at diagnosis than those with *DUOX2* biallelic variants.

Conclusions: We found a high frequency of *TSHR* variants in Chinese patients with CH (12.3%), and 4.9% of cases were caused by *TSHR* biallelic variants. Ten variants were identified as loss-of-function variants. The data suggest that the clinical phenotype of CH patients caused by *TSHR* biallelic variants is relatively mild. Our study expands the *TSHR* variant spectrum and provides further evidence for the elucidation of the genetic etiology of CH.

Key Words: Congenital hypothyroidism, Recessive inheritance, Thyroid-stimulating hormone receptor, Variant, Whole-exome sequencing

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INTRODUCTION

Congenital hypothyroidism (CH) is a disease characterized by impairments in neurodevelopment and physical growth and development owing to dysfunction of the hypothalamic-pituitary-thyroid axis present at birth [1]. CH is the most common congenital endocrine metabolic disease, with a global prevalence of 1/2,000–1/3,000 [1]. With the recent developments in gene sequencing technologies, an increasing number of pathogenic genes related to CH, including genes related to thyroid dysgenesis and dyshormonogenesis, have been reported. Among these, the thyroid-stimulating hormone (TSH) receptor (*TSHR*) gene is one of the widely investigated candidate genes [2, 3].

The human *TSHR* gene is located on chromosome 14q31 and encodes a G-protein-coupled receptor that consists of a seven-transmembrane domain (TMD) and a large extracellular domain (ECD) responsible for high-affinity hormone binding. The TSHR is activated upon binding to TSH and induces two signal transduction pathways: the Gs/cyclic AMP (cAMP) pathway and the Gq/11 phospholipase C pathway, which contribute to thyroglobulin iodination and cell proliferation, whereas the Gs pathway is also responsible for iodine uptake regulation in thyrocytes [4, 5]. Both pathways are important for thyroid hormone synthesis and thyroid development [4, 6].

Loss-of-function (LOF) variants in the *TSHR* can cause TSH resistance, which leads to congenital nongoitrous hypothyroidism 1 (OMIM: 275200), which presents a broad spectrum of phenotypes, ranging from severe congenital hypothyroidism to mild euthyroid hyperthyrotropinemia [2, 7-10]. These LOF variants may result in a thyroid gland of normal position and size or in thyroid dysgenesis [11]. LOF variants in *TSHR* were first described in patients with TSH resistance in 1995 [12]. Up to April 2021, 202 *TSHR* variants have been reported and documented in the Human Gene Mutation Database (HGMD). However, the biological functions of most *TSHR* variants remain unknown, and genotype–phenotype relationships have not yet been clearly established.

We previously identified 15 *TSHR* variants in 13 out of 220 Chinese patients with CH [13]. In the present study, we enrolled an additional 367 patients with CH, expanding the sample for screening *TSHR* variants and characterizing the phenotypes of patients with CH carrying *TSHR* variants. The biological functions of the variants were investigated through a series of *in vitro* experiments. We expected this study to deepen our understanding of the genetic landscape and functional consequences of *TSHR* variants and to provide valuable insights into the clinical management of patients harboring *TSHR* variants.

MATERIALS AND METHODS

Patients

In total, 367 patients were enrolled from the Chinese Han populations in Jiangsu province, Fujian province, Anhui province, and Shanghai. Among them, 362 patients (98.7%) received neonatal CH screening, which was performed using filter-paper blood spots (obtained through a heel prick) within 3–5 days after birth. Patients with TSH levels ≥10 μIU/mL at initial screening were recalled for re-examination using an immune-chemiluminescence assay (UniCelDxI 800; Beckman, Indianapolis, IN, USA) to determine the levels of TSH, free triiodothyronine (FT3), and free thyroxine (FT4). The details of the diagnostic standards for CH have been described in our previous study [14]. In addition, five patients who were on l-thyroxine replacement therapy were recruited from outpatient clinics. Although these patients were not neonatally screened, they had a clear history of CH. Thyroid morphology was determined by experienced radiologists through thyroid ultrasound or technetium-99m scanning. Written informed consent to participate was provided by the participants' legal guardians, and the study was approved by the Ethics Committee of Shanghai Ninth People's Hospital affiliated with Shanghai Jiao Tong University School of Medicine, Shanghai, China (approval number: 2016-76-T33).

Whole-exome sequencing (WES)

WES was performed as previously described [15]. Genomic DNA was extracted from peripheral blood, fragmented to 200–300 bp, and ligated to adapters using the KAPA HyperPrep Kit (Roche, Basel, Switzerland). Exonic hybrid capture was performed according to the instructions in the Roche SeqCap EZ Library SR User's Guide. Library quality and levels were determined using the MAN CLS140145 DNA 1 K Chip (PerkinElmer, Waltham, MA, USA) and the PE LabChip GXII Touch (PerkinElmer, Waltham, MA, USA). The Illumina HiSeq 3000 system (Illumina, San Diego, CA, USA) was then used to sequence the paired-end libraries with 150-bp paired-end reads, averaging approximately 100× depth.

Statistical analysis

IBM SPSS Statistics version 25.0 (IBM Corp., Armonk, NY, USA) was used for all statistical analyses. Quantitative variables are presented as mean±SE. The normality of the data was assessed using the Shapiro–Wilk test and intergroup comparisons

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were performed using Student's unpaired *t*-test (for normally distributed data) or the Mann–Whitney *U* test (for non-normally distributed data), as appropriate. Categorical variables are presented as percentages and were compared using the chi-square test or Fisher exact test, as appropriate. $P < 0.05$ was considered statistically significant.

Additional methods are available in the Supplemental Materials.

RESULTS

Clinical characteristics of 367 patients with CH

In total, 367 patients with CH, including 196 boys and 171 girls, were recruited in this study. The mean serum FT3, FT4, and TSH levels at diagnosis were 4.87 pmol/L, 10.55 pmol/L, and 77.57 μIU/mL, respectively (reference ranges: FT3, 3.85–6.01 pmol/L; FT4, 7.46–21.11 pmol/L; TSH, 0.34–5.60 μIU/mL). Based on the serum FT4 level at diagnosis, CH was classified as severe (FT4 < 5 pmol/L), moderate (5 pmol/L ≤ FT4 < 10 pmol/L), or mild (FT4 ≥ 10 pmol/L) [16]. In the present cohort, 49.1% of patients had moderate or severe CH, and 50.9% of patients presented with mild CH. There were no significant differences in hormone levels and other clinical characteristics between boys and girls (Table 1).

Screening for *TSHR* variants in Chinese patients with CH and pedigree analysis

Among the 367 patients with CH, 45 patients carried 17 nonsynonymous *TSHR* variants, including 16 missense variants and one nonsense variant. The *TSHR* variant frequency was 12.3% (45/367). Out of 17 variants, three (p.S237G, p.W546C, and p.M728T) were first reported in this study, and 10 were recurrent variants (p.G132R, p.G245S, p.S305R, p.N432S, p.R450H, p.F525S, p.R609X, p.Y613C, p.V689G, and p.E758K). p.R450H, which is a hotspot variant in the Chinese population, had the highest frequency (2.7%) (Table 2). Four of the 17 variants were located in the leucine-rich repeat (LRR) domain of the TSHR protein (Fig. 1A). Conservation analysis of the three novel variants showed that p.W546C was highly conserved across species, whereas p.M728T was less conserved (Fig. 1B). Out of the 45 patients with *TSHR* variants, 18 patients carried biallelic variants. We conducted a long-term follow-up of two patients with

Table 1. Clinical characteristics of 367 patients with CH

Clinical characteristics	All patients (N = 367)	Male (N = 196)	Female (N = 171)	*P*
Gestational weeks (weeks)	39.0 ± 0.3 (54)	38.5 ± 0.5 (23)	39.4 ± 0.3 (31)	0.177
Birth weight (kg)	3.3 ± 0.1 (55)	3.3 ± 0.1 (22)	3.3 ± 0.1 (33)	0.241
Birth length (cm)	50.1 ± 0.2 (52)	50.1 ± 0.3 (22)	50.1 ± 0.2 (30)	0.547
Age at diagnosis (days)	20.1 ± 1.0 (190)	20.9 ± 1.5 (102)	19.2 ± 1.2 (88)	0.708
Initial dose (μg)*	30.3 ± 1.2 (94)	29.1 ± 1.8 (38)	31.1 ± 1.6 (56)	0.453
Severity classification				0.445
Mild	113 (50.9%)	58 (48.7%)	55 (53.4%)	
Moderate	54 (24.3%)	33 (27.7%)	21 (20.4%)	
Severe	55 (24.8%)	28 (23.5%)	27 (26.2%)	
Thyroid morphology				0.227
Normal	84 (80%)	39 (86.7%)	45 (75%)	
Goiter	5 (4.8%)	1 (2.2%)	4 (6.7%)	
Orthotopic hypoplasia	15 (14.3%)	4 (8.9%)	11 (18.3%)	
Ectopy	1 (1.0%)	1 (2.2%)	0 (0.0%)	
Biochemical tests at diagnosis				
FT3 (pmol/L)	4.87 ± 0.12 (222)	4.96 ± 0.15 (119)	4.77 ± 0.19 (103)	0.274
FT4 (pmol/L)	10.55 ± 0.45 (222)	10.40 ± 0.59 (119)	10.73 ± 0.70 (103)	0.749
TSH (μIU/mL)	77.57 ± 4.18 (228)	77.09 ± 6.17 (122)	78.12 ± 5.54 (106)	0.631

*Dose of levothyroxine administered for the first time after the diagnosis of CH. Data are shown as N (%) or mean ± SE. The exact number of patients in each group is shown in parentheses after mean ± SE.
Abbreviations: CH, congenital hypothyroidism; FT3, free triiodothyronine; FT4, free thyroxine; TSH, thyroid-stimulating hormone.

Table 2. Detailed information on the *TSHR* variants detected in this study

Genomic position (hg38, chr14)	Exon	rs ID	cDNA change	Amino acid change	N patients (AF)*	AF in public database†
81557414	exon5	rs760874290	c.394G>C	p.G132R	10 (0.0163)	0.00054
81574751	exon8	rs771936985	c.647T>C	p.I216T	1 (0.0014)	0.00022
81606039	exon9	NA	c.709A>G	p.S237G	1 (0.0014)	0
81606063	exon9	rs189506473	c.733G>A	p.G245S	4 (0.0054)	0.00114
81606153	exon9	rs180762551	c.823G>A	p.A275T	1 (0.0014)	0.00033
81609317	exon10	rs142122217	c.915T>A	p.S305R	2 (0.0027)	0.00326
81609697	exon10	rs368268514	c.1295A>G	p.N432S	2 (0.0027)	0.00016
81609751	exon10	rs189261858	c.1349G>A	p.R450H	17 (0.0272)	0.00256
81609976	exon10	rs200138601	c.1574T>C	p.F525S	5 (0.0068)	0.00180
81609978	exon10	rs777308150	c.1576G>A	p.A526T	1 (0.0014)	0.00022
81609993	exon10	rs139892516	c.1591C>T	p.R531W	1 (0.0014)	0.00011
81610040	exon10	NA	c.1638G>C	p.W546C	1 (0.0014)	0
81610227	exon10	rs763679435	c.1825C>T	p.R609X	2 (0.0027)	0
81610240	exon10	rs540799629	c.1838A>G	p.Y613C	2 (0.0027)	0.00054
81610468	exon10	rs761341933	c.2066T>G	p.V689G	3 (0.0041)	0.00054
81610585	exon10	NA	c.2183T>C	p.M728T	1 (0.0014)	0.00016
81610674	exon10	rs746522401	c.2272G>A	p.E758K	4 (0.0054)	0.00033

*Allele frequency in our congenital hypothyroidism sample bank.
†Allele frequency in the gnomAD exome Eastern Asian database.
Abbreviations: *TSHR*, thyroid-stimulating hormone receptor gene; rs ID, reference SNP identification; AF, allele frequency; NA, not applicable.

TSHR biallelic variants (CHT558 and CHT573) and collected blood samples from their parents for pedigree analysis. Sanger sequencing showed that the biallelic variants carried by the patients were inherited from their father and mother separately, which is in line with an autosomal recessive inheritance pattern (Fig. 2).

Pathogenicity prediction of detected *TSHR* variants

The potential effects of the 17 variants identified were assessed using *in silico* programs (SIFT, Polyphen-2, Mutation Taster, and M-CAP). All four programs predicted that the variants p.I216T, p.G245S, p.A275T, p.N432S, p.R450H, p.A526T, p.R531W, p.W546C, p.Y613C, and p.V689G were detrimental to TSHR protein function and that the novel p.M728T variant was harmless. The prediction results of the other variants were inconsistent among the four programs (Supplemental Data Table S1).

Subsequently, we predicted the three-dimensional structure of the wild-type (WT) and three novel mutant proteins using *in silico* tools. In the novel variant p.S237G, a polar neutral serine was replaced with a non-polar aliphatic glycine, disrupting the hydrogen bond between serine 237 and lysine 211 (Supplemental Data Fig. S1A). For the p.W546C variant, the aromatic tryptophan residue at 546 was mutated to a neutral cysteine, disrupting the hydrogen bond between tryptophan 546 and asparagine 455, destabilizing the helix (Supplemental Data Fig. S1B). As for p.M728T, the model confidence at amino acid 738 was very low; therefore, it was not analyzed.

The American College of Medical Genetics (ACMG) issued new guidelines for the interpretation of sequence variants in 2015, describing a process for classifying variants into five categories based on criteria related to typical variant evidence types (such as population data, computational data, functional data, and segregation data). Variants are classified as pathogenic (P), likely pathogenic (LP), variants of uncertain significance (VUS), likely benign (LB), or benign (B) [17]. Based on the available evidence, the pathogenicity of the 17 variants identified was classified according to the ACMG guidelines and standards. Five variants (p.G132R, p.N432S, p.R450H, p.F525S, and p.R609X) were classified as P or LP, and p.M728T was classified as LB. The remaining 11 variants were classified as VUS (Supplemental Data Table S1).

Clinical characteristics of CH patients with *TSHR* variants

The clinical phenotypes of the 45 CH patients with *TSHR* vari-

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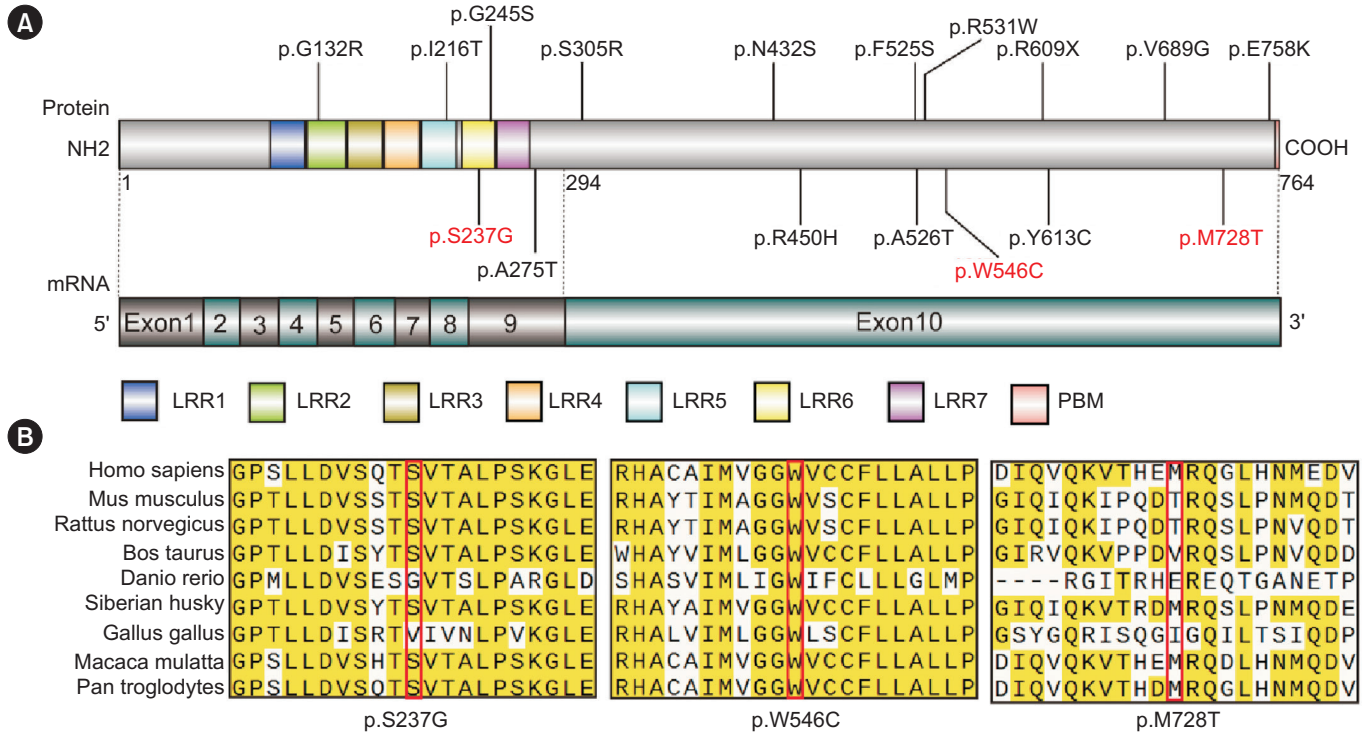

Fig. 1. Location of 17 variants in the TSHR protein and conservation analysis of the three novel variants identified in this study. (A) Distribution of 17 *TSHR* variants identified in the 367 patients with CH. The TSHR comprises seven LRR domains and one (PSD-95/Dlg/ZO-1) PDZ-binding motif (PBM), which is a short linear motif that interacts with a large family of protein–protein interaction domains found in prokaryotes and eukaryotes termed PDZ domains. The upper panel shows a schematic diagram of the TSHR protein sequence, and the lower panel shows a schematic diagram of the corresponding *TSHR* mRNA sequence. Red font denotes novel variants. (B) Conservation analysis of the three novel variants. Amino acid sequences of the TSHR from various species were downloaded from the NCBI website and aligned using the SnapGene software. The mutated amino acids in all TSHR homologs are indicated using red boxes.
Abbreviations: TSHR, thyroid-stimulating hormone receptor; CH, congenital hypothyroidism; LRR, leucine-rich repeat; NCBI, National Centre for Biotechnology Information.

ants were compared with those of the 322 CH patients without *TSHR* variants. There were no significant differences between the two groups in terms of hormone levels, age at diagnosis, and initial levothyroxine dose (Supplemental Data Table S2).

Thyroid functional information at diagnosis was collected for 25 out of 45 patients who harbored *TSHR* variants, including four patients with severe CH, seven with moderate CH, and 14 with mild CH. One patient (CHT241) harboring *TSHR* variants had thyroid dysgenesis (Supplemental Data Table S3). Among the seven patients with *TSHR* biallelic variants, only one patient showed moderate CH, and the remaining six patients presented with mild CH. However, patients with the *TSHR* monoallelic variant can present with mild to severe CH. Surprisingly, we found that the patients with the *TSHR* monoallelic variant had more severe hypothyroidism, with lower FT4 levels (9.58±1.50 vs. 15.87±1.18, $P=0.020$) at diagnosis, than patients with *TSHR* biallelic variants (Fig. 3A–3C).

Dual oxidase 2 (*DUOX2*) is a key protein for thyroid hormone synthesis, and *DUOX2* is the most frequently mutated gene in Chinese patients with CH [14, 18]. We compared the clinical characteristics of patients with *TSHR* or *DUOX2* biallelic variants in the present cohort. Compared with CH patients with *DUOX2* biallelic variants, patients with *TSHR* biallelic variants had lower serum TSH levels and higher FT3 and FT4 levels at diagnosis (TSH: 52.96±17.84 vs. 105.77±5.48, $P=0.012$; FT3: 5.71±0.43 vs. 4.47±0.15, $P=0.025$; FT4: 15.87±1.18 vs. 7.20±0.45, $P<0.001$) (Fig. 3D–3F). In patients with *DUOX2* variants, hypothyroidism may vary with age, whereas in patients with *TSHR* variants, it tends to remain stable over time. Therefore, we compared thyroid function at 6 months and 3 yrs of age between patients carrying *TSHR* biallelic variants and those carrying *DUOX2* biallelic variants. Interestingly, patients harboring *TSHR* biallelic variants exhibited higher FT4 levels at both 6 months and 3 yrs of age than patients carrying *DUOX2* biallelic

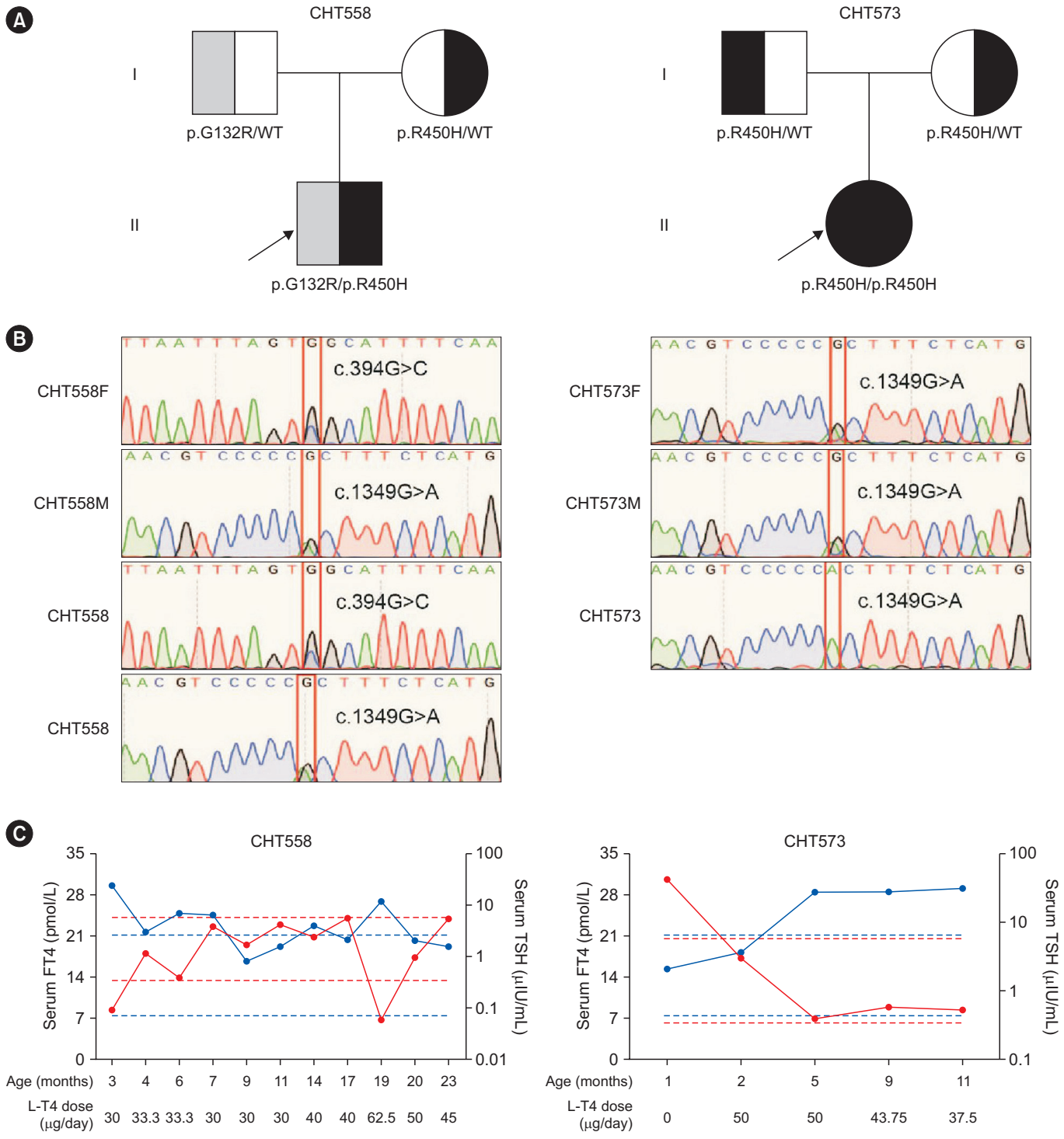

Fig. 2. Pedigree analysis, Sanger validation, and thyroid function follow-up of two patients carrying *TSHR* biallelic variants. (A) Genotypes and pedigrees of two patients with *TSHR* biallelic variants. Roman numerals indicate generations. Squares denote males, and circles denote females. Gray filling represents individuals carrying p.G132R variants, and black filling denotes individuals carrying p.R450H variants. Arrows indicate the probands. The patients' parents were euthyroid, with a normal-sized thyroid gland. (B) Sanger sequencing of the patients and their family members. The red boxes denote variants. F represents father, and M represents mother. (C) Long-term follow-up of thyroid function in two patients (CHT558 and CHT573). The red and blue broken lines represent the dynamic changes in serum TSH and FT4 levels, respectively. The two red and blue dotted lines indicate the upper and lower reference intervals of serum TSH and FT4 levels, respectively.
Abbreviations: TSH, thyroid-stimulating hormone; TSHR, TSH receptor; FT4, free thyroxine; L-T4, levothyroxine.

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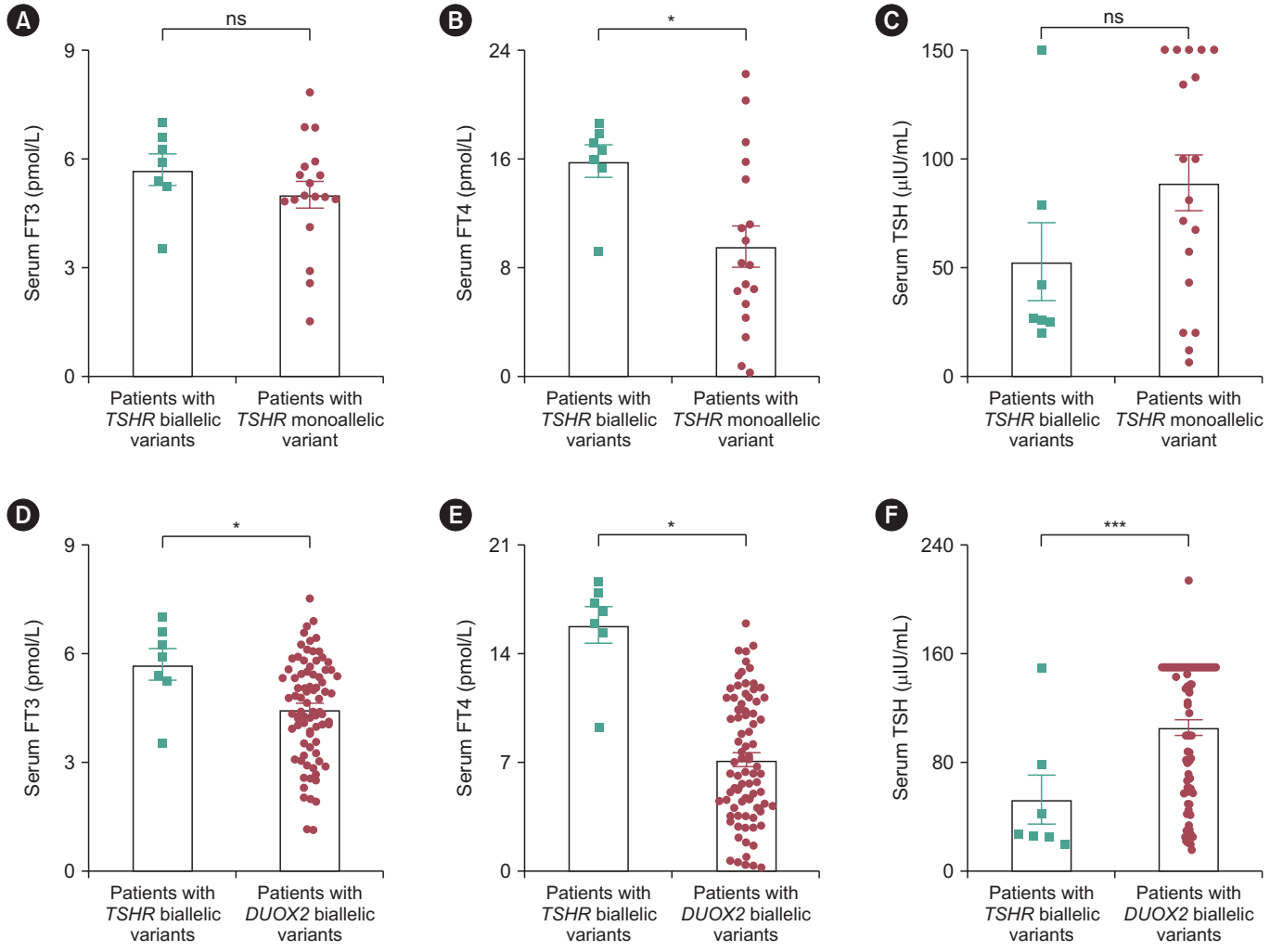

Fig. 3. Comparison of thyroid function at diagnosis. (A–C) Comparison of serum FT3, FT4, and TSH levels at diagnosis between patients with *TSHR* biallelic variants and those with the *TSHR* monoallelic variant. The number of patients carrying *TSHR* biallelic or monoallelic variants is seven and 18, respectively. (D–F) Comparison of serum FT3, FT4, and TSH levels at diagnosis between patients with biallelic *TSHR* or *DUOX2* variants. The number of patients carrying *TSHR* or *DUOX2* biallelic variants is seven and 81, respectively. The Mann–Whitney *U* test was used to compare serum FT4 and TSH levels between the two groups, and serum FT3 levels at diagnosis were compared between the two groups using Student's *t*-test. $*P<0.05$, $***P<0.001$.
Abbreviations: TSH, thyroid-stimulating hormone; TSHR, TSH receptor; FT3, free triiodothyronine; FT4, free thyroxine; ns, no significance.

variants (6 months: 20.60±1.27 vs. 16.77±0.65, $P=0.041$; 3 yrs: 22.72±1.73 vs. 16.83±0.96, $P=0.019$) (Supplemental Data Fig. S2).

Functional assessment of the *TSHR* variants *in vitro*

The eight variants (p.G132R, p.I216T, p.G245S, p.N432S, p.R450H, p.F525S, p.A526T, and p.V689G) detected in 18 patients with *TSHR* biallelic variants and the three novel variants (p.S237G, p.W546C, and p.M728T) were selected for molecular function assessment. The variants were transiently transfected into 293T cells, and Gs/cAMP and Gq/11 signal transduction were investigated by measuring cAMP levels and luciferase activity, respectively. Compared with 293T cells transfected with the WT plasmid, cAMP production in response to bovine TSH (bTSH) was significantly reduced in cells transfected with the p.G132R, p.I216T, p.S237G, p.G245S, p.N432S, p.R450H, p.F525S, p.A526T, and p.W546C mutant plasmids. However, the p.V689G and p.M728T variants did not affect cAMP production (Fig. 4A). The p.G132R, p.I216T, p.S237G, p.G245S, p.F525S, p.A526T, p.W546C, and p.V689G variants showed partial Gq/11 signaling activity (14%–57%), whereas activity was almost abrogated for the p.N432S and p.R450H variants (<10%)

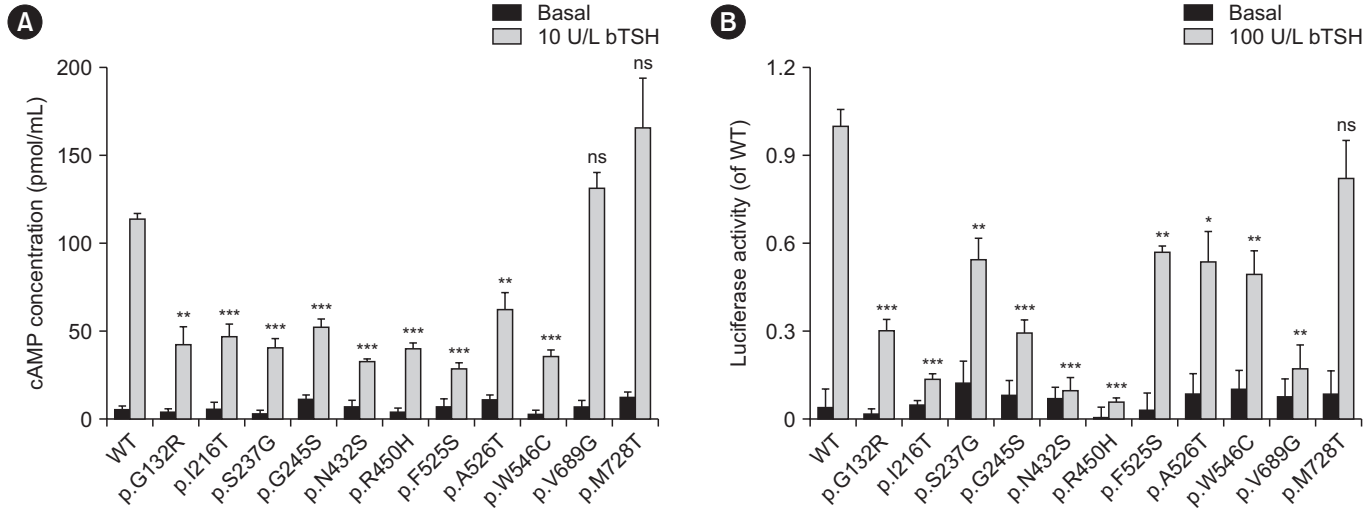

Fig. 4. Signaling properties of WT and 11 *TSHR* variants. (A) cAMP production in 293T cells. 293T cells were transfected with the respective *TSHR* expression vectors (WT or mutant) and incubated with or without 10 U/L bTSH. Intracellular cAMP levels were measured using a cAMP assay kit. Student's *t*-test was used to compare the cAMP levels between the WT and each variant. (B) Gq/11 signaling in cells expressing the *TSHR* variants. Gq/11 signaling in cells expressing the *TSHR* variants was examined indirectly based on firefly luciferase activity in 293T cells. Cells were treated with or without 100 U/L bTSH, and luciferase activity was measured. Student's *t*-test was used to compare luciferase activity between the WT and each variant. Data are representative of three independent experiments (each performed in quadruplicate) with similar results, and the values represent the mean ± SE. Note that there are no significant differences in the levels of intracellular cAMP and Gq/11 activity between the variants and the WT in the basal state. $*P<0.05$, $**P<0.01$, $***P<0.001$.
Abbreviations: WT, wild-type; TSHR, TSH receptor; bTSH, bovine thyroid-stimulating hormone; FT3, free triiodothyronine; FT4, free thyroxine; ns, no significance.

after stimulation with 100 U/L bTSH. The p.M728T variant had no effect on Gq/11 signaling (Fig. 4B).

We next investigated the protein expression and subcellular localization of the three novel variants (p.S237G, p.W546C, and p.M728T) in 293T cells. Western blot analysis showed no significant differences in protein expression between the WT and the three mutants (Supplemental Data Fig. S3A and 3B). Subcellular localization analysis showed that the WT and three mutant TSHR proteins all localized to the cell membrane in an intact manner (Supplemental Data Fig. S3C).

DISCUSSION

Through comprehensive screening, we identified 17 distinct *TSHR* variants in 367 CH patients in China. We found a high frequency of *TSHR* variants in Chinese patients with CH (45/367, 12.3%), with 4.9% of patients carrying biallelic *TSHR* variants. We identified three novel variants (p.S237G, p.W546C, and p.M728T), two of which (p.S237G and p.W546C) impaired TSHR biological functions in the Gs/cAMP and Gq/11 pathways.

Seventeen non-synonymous *TSHR* variants were identified in 45 CH patients, with a detection rate of 12.3%, which is higher than the rates reported in most domestic studies [13, 19-21] but lower than those in two cohort studies in Italy and Korea [22, 23]. Most *TSHR* LOF variants reported to date are located in exons 1, 4, 6, and 10 [11]. In this study, 12 of the 17 identified variants were located in exon 10, whereas none were located in exons 1, 4, and 6. These findings suggest that there may be regional and ethnic differences in the spectrum of *TSHR* variants. In addition, we found a hotspot variant, p.R450H, which is one of the most common *TSHR* LOF variants and has been demonstrated to have a founder effect in Japan [24].

Notably, among the 45 patients carrying *TSHR* variants, 18 carried biallelic variants. The total residual Gs/cAMP and Gq/11 pathway signaling activities in CH patients with *TSHR* biallelic variants were calculated as the sum of pathway signaling activities from both *TSHR* variant alleles divided by two. Two patients (CHT385 and CHT573) harboring the p.R450H homozygous variant, who had 35% Gs/cAMP signaling pathway activity and 6% Gq/11 signaling pathway activity, had clinically similar phenotypes and presented with mild hypothyroidism. Patient CHT506, who carried the p.G132R homozygous variant, had a 62% reduction in Gs/cAMP signaling pathway activity and 70% Gq/11 signaling pathway activity and was diagnosed as having

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moderate CH, with serum TSH and T4 levels of 150.00 μIU/mL and 9.27 pmol/L, respectively. Patient CHT436, who harbored the p.N432S/p.R450H biallelic variants with residual Gs/cAMP and Gq/11 signaling pathway activities of 32% and 8%, respectively, presented with mild CH, with serum TSH and FT4 levels of 27.26 μIU/mL and 18.66 pmol/L. Patients CHT445 and CHT516 carried the p.G132R/p.R450H biallelic variants, with residual Gs/cAMP and Gq/11 signaling pathway activities of 37% and 18%, respectively. They both exhibited mild CH. Patient CHT553 harboring the p.R450H/p.F525S biallelic variants had 30% and 32% residual Gs/cAMP and Gq/11 signaling pathway activities, respectively, and was diagnosed as having mild CH, with serum TSH and FT4 levels of 25.56 μIU/mL and 15.96 pmol/L, respectively (Supplemental Data Table S4). These functional experimental results in patients with *TSHR* biallelic variants support the hypothesis that *TSHR* variants can cause the onset of CH. Pedigree analysis of two patients showed that CH caused by *TSHR* variants is inherited in an autosomal recessive manner, which is consistent with previous findings [12, 25, 26].

LOF *TSHR* variants result in variable TSH resistance manifested as euthyroid hyperthyrotropinemia with a normal thyroid gland (fully compensated TSH resistance), mild hypothyroidism with a normal thyroid gland (partially compensated TSH resistance), or severe hypothyroidism with thyroid dysgenesis (uncompensated TSH resistance) [2]. In the present study, out of seven patients with *TSHR* biallelic variants, six patients presented with mild hypothyroidism, and only one patient presented with moderate hypothyroidism. Moreover, patients with *TSHR* biallelic variants had milder hypothyroidism than those with *DUOX2* biallelic variants. These findings indicate that the phenotypes of CH caused by *TSHR* defects are milder and associated with completely or partially compensated TSH resistance.

The TSHR is a G-protein-coupled receptor with a TMD domain and a large ECD, which comprises an LRR domain involved in hormone binding specificity and a hinge region, linking the LRR domain to the TMD [11, 27]. TSHR activation results in intracellular signaling via the Gs protein, which leads to cAMP cascade activation, and via the Gq protein, which leads to phospholipase C cascade activation. In the present study, four variants (p.G132R, p.I216T, p.S237G, and p.G245S) were located in the LRR domain, and they caused varying degrees of impairment to the Gs/cAMP and Gq/11 signaling pathways. The novel p.S237G variant had no effect on the expression and membrane localization of the TSHR protein but partially hindered Gs/cAMP and Gq/11 signaling. This may be attributed to the replacement of amino acids altering the TSHR protein structure, thereby decreasing its ability to bind to TSH. The novel p.W546C variant, located in the fourth TMD of TSHR, is highly conserved among species. *In silico* tools predicted that this missense variant is detrimental to protein stability and function. *In vitro* experiments demonstrated that the p.W546C variant damages receptor function by affecting the Gs/cAMP and Gq/11 signaling pathways.

p.M728T, another novel variant identified in the present study, is located in the C-terminal intracellular region of TSHR. Chazenbalk, *et al*. [28] confirmed that the removal of the C-terminal 2/3 residues (Q709–L764) of TSHR did not impair receptor function. Concurrently, functional experiments in the present study showed that the p.M728T variant did not interfere with the Gs/cAMP or Gq/11 pathway. Numerous studies have confirmed that the hotspot variant p.R450H not only results in reduced cAMP activity and severely impaired Gq/11 pathway activity but also reduces the TSH binding ability of TSHR [5, 13, 24, 29], which is consistent with our findings.

The phenotypes of the *TSHR* monoallelic variant are reportedly always mild, whereas biallelic variants are often associated with a more severe phenotype [7]. However, we found that patients with the *TSHR* monoallelic variant presented with mild to severe CH. Therefore, we compared thyroid function at diagnosis in patients with monoallelic and biallelic *TSHR* variants. Surprisingly, patients with the *TSHR* monoallelic variant had lower FT4 levels, which may be because of the following reasons. First, in our cohort, 12 of 27 patients with the *TSHR* monoallelic variant harbored biallelic variants in 21 other CH pathogenic genes (*NKX2-1*, *NKX2-5*, *FOXE1*, *PAX8*, *HHEX*, *TPO*, *SLC5A5*, *TG*, *DUOX2*, *DUOXA2*, *TSHR*, *SLC26A4*, *IYD*, *DIO1*, *DIO2*, *THRA*, *THRB*, *DUOX1*, *DUOXA1*, *GNAS*, and *SLC16A2*) as described in our previous study [14] (Supplemental Data Table S3). Compared with patients with *TSHR* biallelic variants, patients with oligogenic variants, including in *TSHR*, had lower FT4 levels and higher TSH levels at diagnosis, whereas patients with only a *TSHR* monoallelic variant showed no difference in thyroid function at diagnosis (Supplemental Data Fig. S4), which partially explains why patients with the *TSHR* monoallelic variant presented more severe hypothyroidism than those with *TSHR* biallelic variants. Second, the genetic etiology of CH is largely unknown, and patients with the *TSHR* monoallelic variant may also carry novel CH-causative genes, leading to a more severe phenotype. Finally, environmental modifiers, such as iodine intake and ethnicity, should be considered in addition to genetic factors to explain this phenotypic variation. For example, Vigone, *et al*. [30] reported phenotypic differences between two brothers harboring the same genetic variants attributed to different neonatal iodine

supplies, which suggested that the different neonatal iodine supplies acted as disease modifiers.

This study had some limitations. First, among the 18 patients carrying biallelic *TSHR* variants, pedigree analysis was conducted for only two families. Second, we did not clarify whether patients carrying *TSHR* variants require lifelong thyroxine therapy. Third, the pathogenic mechanism related to the presence of a heterozygous sequence variant in *TSHR* in patients with CH was not fully identified. In future work, we will mine and analyze unknown pathogenic genes in CH to gain insight into the molecular mechanisms of CH pathogenesis.

In conclusion, we reported 17 *TSHR* variants in 367 Chinese patients with CH and investigated the biological function of 11 variants (eight biallelic and three novel variants). Two novel variants (p.S237G and p.W546C) impair TSHR protein biological function by interfering with Gs/cAMP and Gq/11 signaling. Characterization of the phenotypes of patients with *TSHR* variants revealed that *TSHR* biallelic variants cause mild CH. The present study expanded the *TSHR* variant spectrum and provided further evidence for the elucidation of the genetic etiology of CH.

SUPPLEMENTARY MATERIALS

Supplementary materials can be found via https://doi.org/10.3343/alm.2023.0337

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AUTHOR CONTRIBUTIONS

Zhang HY performed the experiments, analyzed the data, prepared figures and tables, and wrote the paper. Wu FY and Li XS collected clinical samples, analyzed the clinical data, and prepared tables. Tu PH, Zhang CX, and Yang RM conducted the genetic and bioinformatics analyses. Cui RJ and Wu CY conducted the three-dimensional analyses and interpreted the data. Fang Y and Yang L collected clinical samples. Song HD and Zhao SX proposed the study design, recruited the patients, and edited the manuscript. All authors have read and approved the final manuscript.

CONFLICTS OF INTEREST

None declared.

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