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

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SUPPLEMENTAL METHODS

Thyroid-stimulating hormone receptor (TSHR) variant calling from whole-exome sequencing (WES) data

The WES data were processed and analyzed as described in our previous study [1]. First, the Burrows–Wheeler Aligner was used to align the Illumina reads to the human reference genome (hg19). After alignment, only uniquely mapped single reads or confidently mapped paired-end reads were retained. Variants, including single-nucleotide variants and insertions/deletions, were realigned using the Genome Analysis Toolkit software version 2.7-2 (Broad, Boston, MA, USA). Credible variants were identified according to previously described criteria [1]. All variants were validated using Sanger sequencing.

TSHR variant interpretation

The SnapGene software (Dotmatics, Boston, MA, USA, https://www.snapgene.com/) was used to align TSHR protein sequences from different species that were acquired from the National Centre for Biotechnology Information (NCBI) website. The *in silico* programs SIFT [2], PolyPhen-2 [3], Mutation Taster [4], M-CAP [5], and Revel [6] were used to evaluate the effect of the *TSHR* variants identified. The monomer model in AlphaFold version 2.3.1 (Deepmind, London, UK) was used to predict the structure of wild-type (WT) and mutant TSHR proteins [7]. Theoretical modeling of the protein structures was performed using PyMOL version 2.4 (Schrödinger, New York, NK, USA). The pathogenicity of each variant was classified following the standards described by the American College of Medical Genetics (ACMG) [8].

Plasmid construction

Human WT cDNA encoding TSHR was cloned into the p-enhanced green fluorescent protein-N2 (TSHR-pEGFP-N2) plasmid (TransGen Biotech, Beijing, China). Eleven *TSHR* variants (p.G132R, p.I216T, p.S237G, p.G245S, p.N432S, p.R450H, p.F525S, p.A526T, p.W546C, p.V689G, and p.M728T) were introduced into the TSHR-pEGFP-N2 WT plasmid using site-directed mutagenesis (TransGen Biotech). Mutagenesis was confirmed using Sanger sequencing.

Cell culture and transfection

293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA) at 37 °C in a humidified atmosphere containing 5% CO_2. Transient transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Briefly, 293T cells were plated in 24- or 96-well plates and cultured overnight. Subsequently, the cells were transfected with plasmid DNA and cultured in opti-MEM medium without FBS for 4 – 6 hrs. The culture medium was then replaced with fresh DMEM containing 10% FBS.

Cyclic (c)AMP determination and dual-luciferase reporter assay

The Gs/cAMP signaling pathway is activated after the TSHR binds to TSH, which leads to intracellular cAMP accumulation, and the TSHR is capable of stimulating the Gq/11 phospholipase C pathway at higher levels of TSH [9]. For cAMP determination, 293T cells were incubated in Krebs–Ringer phosphate buffer at 37 °C for 15 mins after transfection for 48 hrs. Subsequently, 0 or 10 IU/L bovine TSH (bTSH) (Sigma-Aldrich) was added, and cells were incubated at 37 °C for 1 hr. Cell lysates were prepared, and cAMP levels were measured

using the cAMP assay kit (R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions.

The nuclear factor of activated T cells (NFAT) is a family of transcription factors whose activity is regulated by calcium-dependent calmodulin phospholipase C. Gq/11 phospholipase C signaling in the cells expressing the *TSHR* variants was examined indirectly based on the firefly luciferase activity of 293T cells, which is regulated by the NFAT response element promoter of the reporter plasmid co-transfected with the *TSHR*-containing constructs. 293T cells were seeded into 96-well plates (1×10^4 cells/well) and cultured overnight. Subsequently, the cells were transfected with 100 ng of plasmid DNA per well (20 ng of pEGFP-N2 containing WT, mutant *TSHR,* or empty pEGFP-N2; 8 ng of Renilla luciferase reporter plasmid; and 72 ng of firefly luciferase reporter plasmid). After transfection for 48 hrs, the cells were incubated in DMEM with or without 100 IU/L bTSH at 37 °C for 6 hrs. Firefly and Renilla luminescence intensities of the cells were detected in black flat-bottom 96-well plates using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's protocol.

Western blot and immunofluorescence analyses

For western blot analysis, whole-cell lysates of transiently transfected 293T cells were subjected to 8% sodium dodecyl-sulfate polyacrylamide gel electrophoresis, and western blot analysis was performed using anti-TSHR and anti-GAPDH antibodies (both from Invitrogen). Immunoreactive proteins were visualized using an Odyssey CLx infrared imaging system (LI-COR, Lincoln, NE, USA), and band densities were determined using the Fiji open-source software for processing and analyzing scientific images (https://imagej.net/software/fiji/).

Immunofluorescence analysis of protein localization was performed as previously described [10]. 293T cells were transfected with WT or mutant TSHR-pEGFP-N2 plasmids for 48 hrs. Cell membranes were labeled with the DiI fluorescent probe (Beyotime Biotech, Shanghai, China), and nuclei were stained with 4′,6-diamidino-2-phenylindole (Beyotime Biotech). The GFP fusion protein represented the localization of WT or mutant TSHR proteins. Images were acquired using a confocal microscope (Nikon A1 Microsystems, Tokyo, Japan).

Supplemental Data Table S1. *In silico* prediction of the effects of and ACMG classification of the *TSHR* variants detected in this study

Variant	SIFT	PolyPhen-2	Mutation taster	M-CAP	Revel score	Evidence of ACMG classification *	ACMG classification
p.G132R	Tolerated	Benign	Disease-causing	Possibly pathogenic	0.703	PS3_Supporting, PM1, PM3, PP3	LP
p.I216T	Damaging	Probably damaging	Disease-causing	Possibly pathogenic	0.86	PS3_Supporting, PP3	VUS
p.S237G	Tolerated	Benign	Disease-causing	Possibly pathogenic	0.207	PS3_Supporting, PM2_Supporting	VUS
p.G245S	Damaging	Probably damaging	Disease-causing	Possibly pathogenic	0.882	PS3_Supporting, PP3	VUS
p.A275T	Damaging	Probably damaging	Disease-causing	Possibly pathogenic	0.72	PM3, PP3	VUS
p.S305R	Tolerated	Possibly damaging	Polymorphism	Likely benign	0.219	BS1	VUS
p.N432S	Damaging	Probably damaging	Disease-causing	Possibly pathogenic	0.932	PS3_Supporting, PM1, PM5, PP3	LP
p.R450H	Damaging	Probably damaging	Disease-causing	Possibly pathogenic	0.943	PS3_Supporting, PM1, PM3, PP1, PP3	LP
p.F525S	Tolerated	Probably damaging	Disease-causing	Possibly pathogenic	0.423	PS3_Supporting, PM1, PM3, PM5	LP
p.A526T	Damaging	Probably damaging	Disease-causing	Possibly pathogenic	0.681	PS3_Supporting, PM1	VUS
p.R531W	Damaging	Probably damaging	Disease-causing	Possibly pathogenic	0.568	PM1	VUS
p.W546C	Damaging	Probably damaging	Disease-causing	Possibly pathogenic	0.839	PS3_Supporting, PM1, PM2_Supporting, PP3	VUS
p.R609X	NA	NA	Disease-causing	NA	NA	PVS1_Strong, PM1, PM2_Supporting, PM3, PP1	P
p.Y613C	Damaging	Probably damaging	Disease-causing	Possibly pathogenic	0.488	PM1	VUS
p.V689G	Damaging	Probably damaging	Disease-causing	Possibly pathogenic	0.972	PS3_Supporting, PP3	VUS
p.M728T	Tolerated	Benign	Polymorphism	Likely benign	0.069	BS3_Supporting, BP4	LB
p.E758K	Damaging	Benign	Polymorphism	Possibly pathogenic	0.226	NA	VUS

*The pathogenicity of each variant was classified following the standards described by the American College of Medical Genetics (ACMG) [8]. According to our experimental results *in vitro*, we added the functional evidence based on the guidelines of the functional evidence PS3/BS3 criterion using the ACMG/AMP sequence variant interpretation framework [11].

Abbreviations: SIFT, sorting intolerant from tolerant; PolyPhen-2, Polymorphism Phenotyping v2; M-CAP, Mendelian Clinically Applicable Pathogenicity; Revel, rare exome variant ensemble learner; NA, not applicable; P, pathogenic; LP, likely pathogenic; VUS, variant of uncertain significance; LB, likely benign.

Supplemental Data Table S2. Clinical characteristics of CH patients with or without *TSHR* variants

Clinical characteristics	***TSHR* variant**	***Non-TSHR* variant**	***P***
Sex			0.530
Male	26 (57.8%)	170 (52.8%)	
Female	19 (42.2%)	152 (47.2%)	
Total	45	322	
Biochemical tests at diagnosis			
FT3 (pmol/L)	5.21 ± 0.29 (25)	4.83 ± 0.13 (197)	0.249
FT4 (pmol/L)	11.34 ± 1.25 (25)	10.45 ± 0.48 (197)	0.372
TSH (μIU/mL)	78.92 ± 10.74 (25)	77.40 ± 4.51 (203)	0.703
Age at diagnosis (days)	20.24 ± 2.10 (21)	20.08 ± 1.04 (169)	0.639
Initial dose (μg)	29.17 ± 2.64 (6)	30.40 ± 1.27 (88)	0.910

Data are presented as N (%) or mean ± SE. The exact number of patients in each group is shown in parentheses after mean ± SE.

Abbreviations: CH, congenital hypothyroidism; *TSHR*, thyroid-stimulating hormone receptor gene; FT3, free triiodothyronine; FT4, free thyroxine; TSH, thyroid-stimulating hormone.

Supplemental Data Table S3. Clinical, biological, and genetic characteristics of the 45 CH patients with *TSHR* variants

Case No.	Sex	Birth date	Age at diagnosis (days)	Thyroid function at diagnosis			Severity	*TSHR* variant	Biallelic variants in other known pathogenic genes of CH
				FT3 (3.85 – 6.01) pmol/L	FT4 (7.46 – 21.11) pmol/L	TSH (0.34 – 5.60) µIU/mL			
CHT241*	Female	2017/10/5	23	4.86	8.38	150.00	Moderate	p.F525S	No
CHT247	Male	2017/12/26	23	2.92	2.90	100.00	Severe	p.R450H	No
CHT284	Male	2019/02	NA	NA	NA	NA	NA	p.R609X	DUOX2 (p.E1469K/p.K530X); TG (p.R2455H/p.N2616I)
CHT298	Male	2017/11	15	5.93	15.83	20.28	Mild	p.Y613C	No
CHT301	Male	2018/06	10	5.54	6.44	150.00	Moderate	p.E758K	DUOX2 (p.K530X/p.S199Wfs*122)
CHT302	Male	2018/06	14	4.96	8.24	150.00	Moderate	p.E758K	DUOX2 (p.K530X/p.S199Wfs*122)
CHT322	Male	2016/09	4	4.84	6.31	134.26	Moderate	p.G245S	DUOX2 (c.3693+1G>T/p.K530X)
CHT329	Male	2019/08	NA	NA	NA	NA	NA	p.R450H/p.R450H	No
CHT340	Male	2019/01	17	2.59	4.38	150.00	Severe	p.Y613C	No
CHT343	Male	2019/10	NA	4.94	22.27	6.86	Mild	p.S237G	No
CHT367	Female	2018/12/30	22	1.52	0.33	100.00	Severe	p.S305R	No
CHT374	Female	2019/1/14	15	6.87	0.81	67.66	Severe	p.E758K	No
CHT379	Female	2018/11/1	NA	NA	NA	NA	NA	p.G245S	DUOX2 (p.A1123T/p.R885Q)
CHT384†	Male	2019/01	NA	NA	NA	NA	NA	p.M728T	No
CHT385	Female	2019/09	4	7.01	16.73	26.42	Mild	p.R450H/p.R450H	No
CHT386	Male	2019/02	27	4.99	10.04	71.71	Mild	p.W546C	DUOX2 (p.R885L/p.L320P)
CHT399	Male	2018/06	10	5.78	6.82	150.00	Moderate	p.E758K	DUOX2

									(p.K530X/p.S199Wfs*122)
CHT401	Male	2018/11	NA	NA	NA	NA	NA	p.G132R/p.A526T	No
CHT405	Female	2017/06	20	5.54	14.54	43.26	Mild	p.R450H	No
CHT409	Female	2014	NA	NA	NA	NA	NA	p.G132R/p.G132R	No
CHT413	Male	2019/03	24	4.13	11.20	81.08	Mild	p.F525S	DUOX2 (p.Q570X/p.Q202Tfs*99)
CHT421	Female	2018/12	NA	NA	NA	NA	NA	p.A275T	No
CHT423	Male	2017/03	NA	NA	NA	NA	NA	p.V689G	No
CHT424	Female	2015	NA	NA	NA	NA	NA	p.V689G	No
CHT436	Male	2013/02	18	6.61	18.66	27.26	Mild	p.N432S/p.R450H	No
CHT440	Female	2019/11	30	4.90	20.33	20.24	Mild	p.R450H	No
CHT445	Female	2019/11	20	5.91	17.89	20.42	Mild	p.G132R/p.R450H	No
CHT446	Male	2014	NA	NA	NA	NA	NA	p.G132R/p.R450H	No
CHT449	Male	2018/01	NA	NA	NA	NA	NA	p.R609X	DUOX2 (p.L1343F/p.R683L/p.K530X)
CHT450	Female	2010	NA	NA	NA	NA	NA	p.R531W	DUOX2 (p.L1343F/p.S1237dup/p.R683L)
CHT465	Male	2016	NA	NA	NA	NA	NA	p.G132R/p.N432S	No
CHT466	Female	2017	NA	NA	NA	NA	NA	p.F525S	No
CHT490	Male	2017/01	NA	NA	NA	NA	NA	p.R450H/p.F525S	No
CHT498	Female	2019/12	24	6.90	10.94	57.42	Mild	p.G245S	DUOX2 (c.3693+1G>T/p.R411K)
CHT505	Female	2019/04	45	5.33	5.41	137.38	Moderate	p.S305R	DUOX2 (p.K530X/p.K530X)
CHT506	Male	2019/03	NA	3.53	9.27	150.00	Moderate	p.G132R/p.G132R	No
CHT510	Male	2013	NA	NA	NA	NA	NA	p.G132R/p.R450H	No

CHT516	Male	2017/12	28	5.39	17.25	78.77	Mild	p.G132R/p.R450H	No
CHT521	Female	2019/09	NA	NA	NA	NA	NA	p.G245S/p.V689G	No
CHT531	Male	2009	NA	NA	NA	NA	NA	p.I216T/p.R450H	No
CHT536	Male	2019/10	NA	7.84	17.25	12.13	Mild	p.R450H	No
CHT543	Female	2018/06	NA	NA	NA	NA	NA	p.G132R/p.R450H	No
CHT553	Female	2019/03	NA	6.25	15.96	25.56	Mild	p.R450H/p.F525S	DUOX2 (p.L1343F/p.R683L)
CHT558	Male	2019/11/20	NA	NA	NA	NA	NA	p.G132R/p.R450H	No
CHT573	Female	2019/12	32	5.25	15.34	42.33	Mild	p.R450H/p.R450H	No

*The patient had thyroid dysgenesis.

†The patient's TSH level at newborn screening was 20.68 μIU/mL.

Accession numbers of related genes: *TSHR*: NM_000369.2; *DUOX2*: NM_014080.4; and *TG*: NM_003235.4.

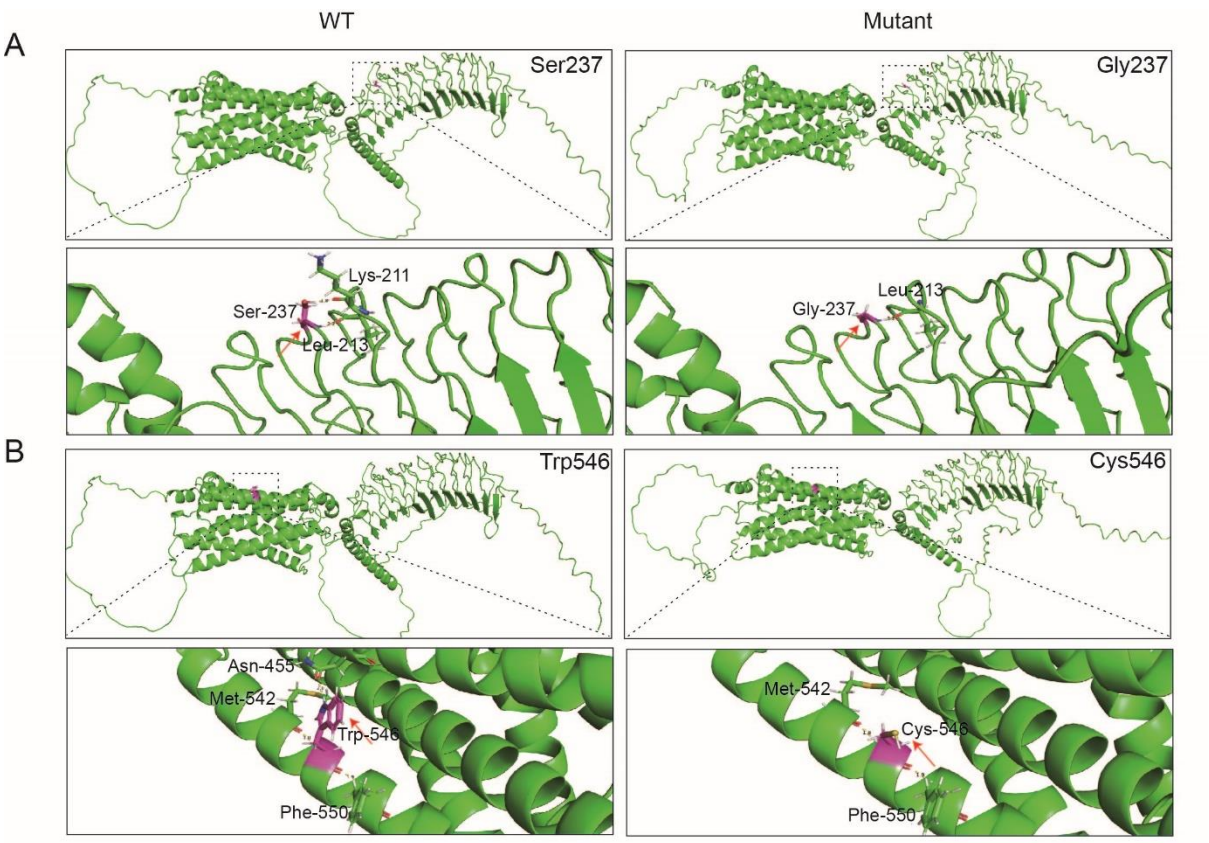
Abbreviations: CH, congenital hypothyroidism; *TSHR*, thyroid-stimulating hormone receptor gene; NA, not available; *DUOX2*, dual oxidase 2 gene; *TG*, thyroglobulin gene.

Supplemental Data Table S4. Genotype–phenotype relationships in seven patients with *TSHR* biallelic variants

Case No.	Sex	Thyroid function at diagnosis			Severity	*TSHR* variants	Gs/cAMP activity (of WT, 100%)			Gq/11 activity (of WT, 100%)		
		FT3 (3.85 – 6.01) pmol/L	FT4 (7.46 – 21.11) pmol/L	TSH (0.34 – 5.60) μIU/mL			Overall residual Gs/cAMP activity *	Variant of allele 1	Variant of allele 2	Overall residual Gq/11 activity *	Variant of allele 1	Variant of allele 2
CHT385	Female	7.01	16.73	26.42	Mild	p.R450H/p.R450H	35	35	35	6	6	6
CHT436	Male	6.61	18.66	27.26	Mild	p.N432S/p.R450H	32	29	35	8	10	6
CHT445	Female	5.91	17.89	20.42	Mild	p.G132R/p.R450H	37	38	35	18	30	6
CHT506	Male	3.53	9.27	150.00	Moderate	p.G132R/p.G132R	38	38	38	30	30	30
CHT516	Male	5.39	17.25	78.77	Mild	p.G132R/p.R450H	37	38	35	18	30	6
CHT553	Female	6.25	15.96	25.56	Mild	p.R450H/p.F525S	30	35	25	32	6	57
CHT573	Female	5.25	15.34	42.33	Mild	p.R450H/p.R450H	35	35	35	6	6	6

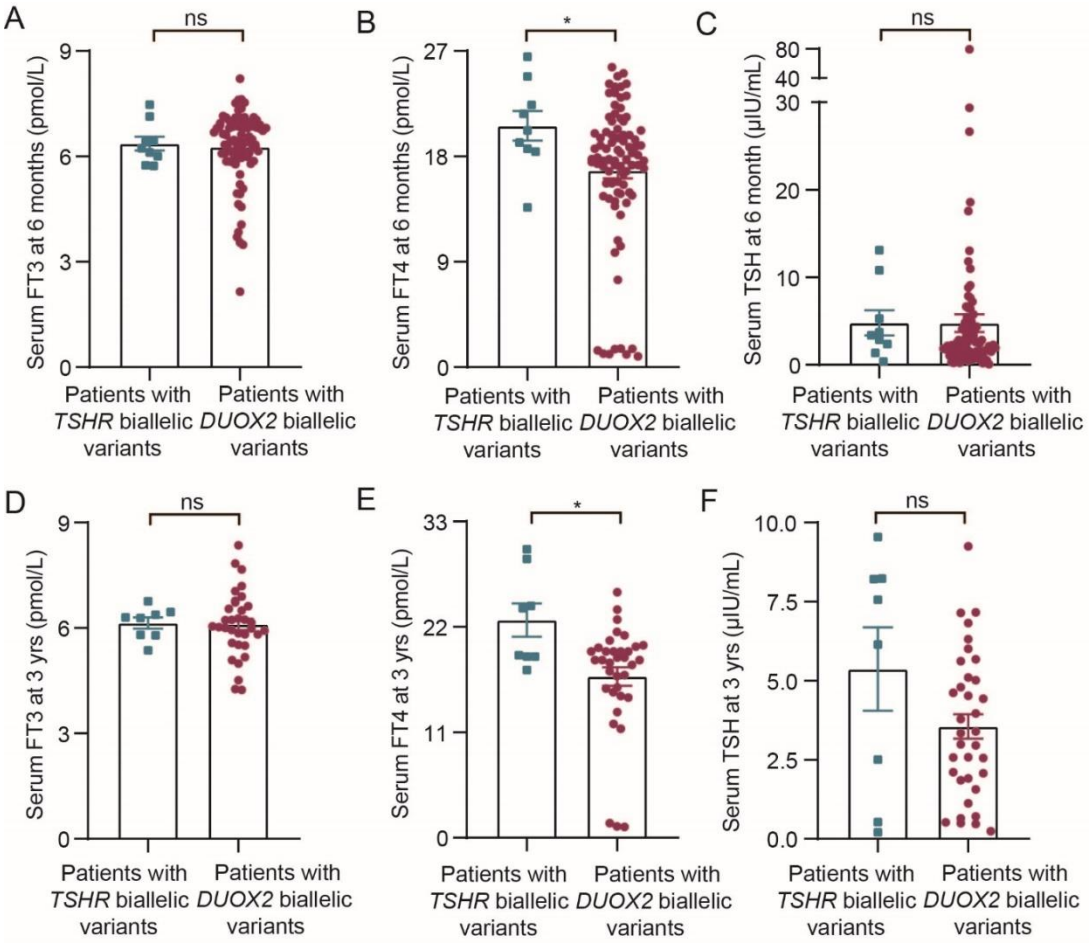
*The overall residual Gs/cAMP and Gq/11 pathway signaling activities in patients with congenital hypothyroidism with *TSHR* biallelic variants were calculated as the sum of the pathway signaling activities from both alleles of the *TSHR* variants divided by two.

Abbreviations: *TSHR*, thyroid-stimulating hormone receptor gene; FT3, free triiodothyronine; FT4, free thyroxine; TSH, thyroid-stimulating hormone; WT, wild-type.



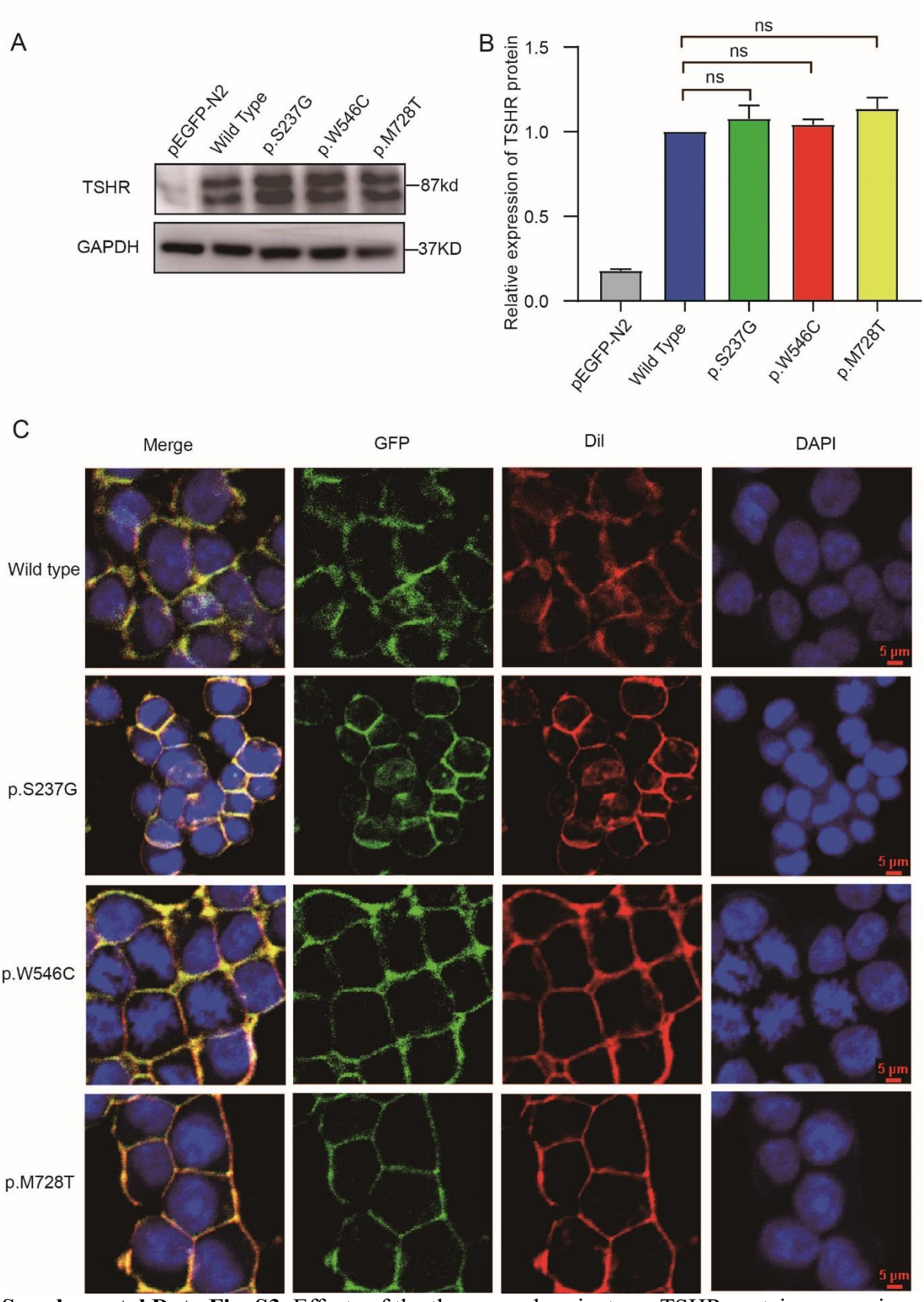
Supplemental Data Fig. S1. Three-dimensional structure models of the WT and two novel mutant TSHR proteins. (A) p.S237G. (B) p.W546C. The red arrows indicate the location of the amino acid residues under study, and a magnified view of the region is shown below the model. The yellow dotted lines represent hydrogen bonds between amino acid residues, and inter-atomic distances are presented in Ångström.

Abbreviations: WT, wild-type; TSHR, thyroid-stimulating hormone receptor.



Supplemental Data Fig. S2. Comparison of thyroid function between patients with *TSHR* or *DUOX2* biallelic variants. (A–C) Comparison of serum FT3, FT4, and TSH levels at 6 months of age between patients with *TSHR* biallelic variants and those with *DUOX2* biallelic variants. The numbers of patients carrying *TSHR* biallelic or *DUOX2* biallelic are nine and 86, respectively. The Mann–Whitney U test was used to compare the serum FT3, FT4, and TSH levels between the two groups. (D–F) Comparison of serum FT3, FT4, and TSH levels at the age of 3 yrs between patients with *TSHR* biallelic variants and those with *DUOX2* biallelic variants. The numbers of patients carrying *TSHR* biallelic or *DUOX2* biallelic are eight and 35, respectively. The Mann–Whitney U test was used to compare the serum FT4 levels between the two groups, and serum FT3 and TSH levels at 3 yrs of age were compared using Student's *t*-test. *$P < 0.05$, ns, no significance.

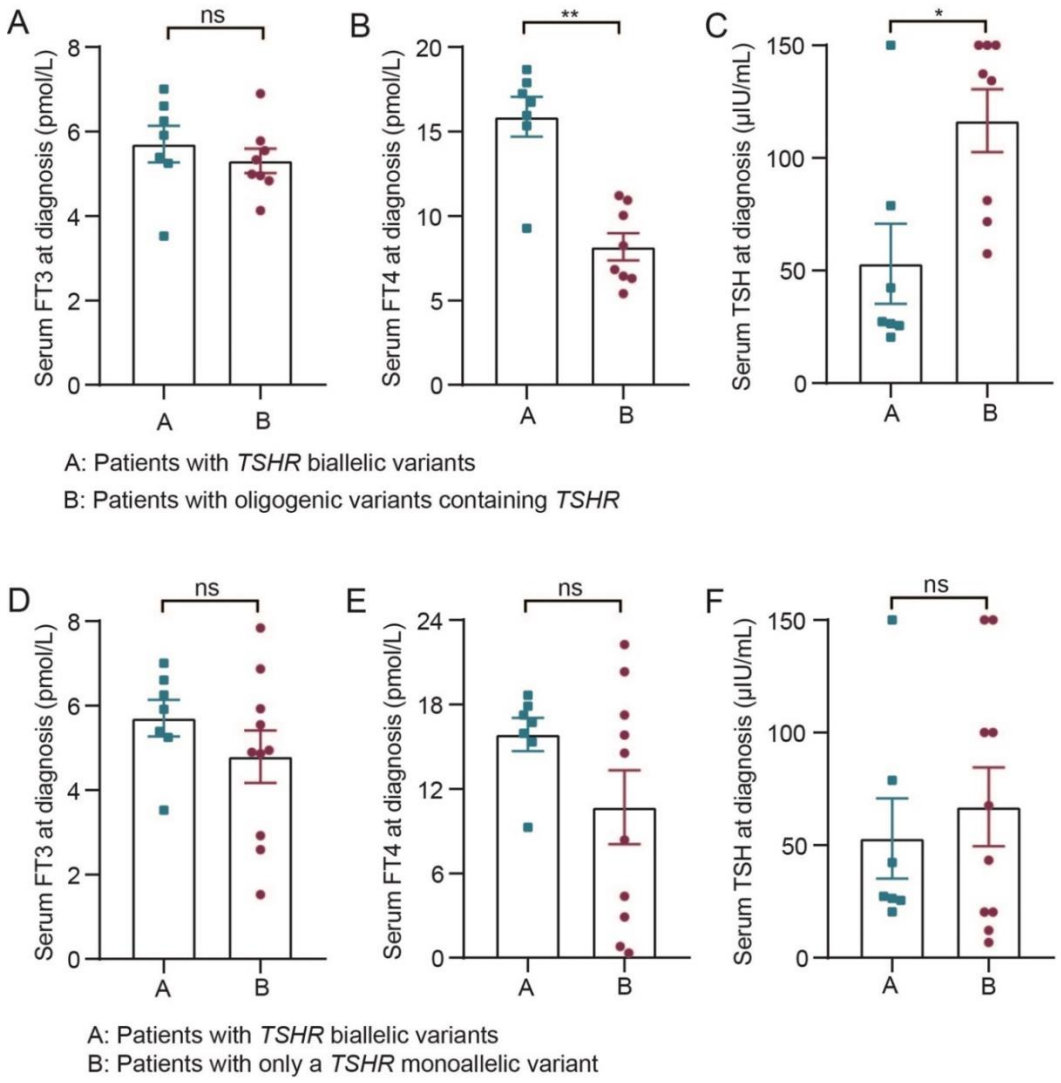
Abbreviations: *TSHR*, thyroid-stimulating hormone receptor gene; FT3, free triiodothyronine; FT4, free thyroxine; TSH, thyroid-stimulating hormone; *DUOX2*, dual oxidase 2 gene.



Supplemental Data Fig. S3. Effects of the three novel variants on TSHR protein expression and subcellular localization. (A) Representative western blot image of WT and mutant TSHR

proteins. (B) Relative protein expression levels of the three novel variants in comparison to the WT TSHR protein. The relative protein level in each sample was normalized to GAPDH expression and is expressed as a percentage of WT TSHR protein. The relative protein level of WT TSHR was set as 100%. ns, no significance. (C) Subcellular localization of the three novel variants in 293T cells. C-terminal green fluorescent protein (GFP) fusion protein denotes the localization of WT or mutant TSHR proteins (green). The cell membranes were labeled with the DiI fluorescent probe (red), and nuclei were stained with 4′,6-diamidino-2-phenylindole (blue). Scale: 5 μm.

Abbreviations: TSHR, thyroid-stimulating hormone receptor; WT, wild-type.



Supplemental Data Fig. S4. Comparison of thyroid function at diagnosis between patients with *TSHR* monoallelic variants and those with biallelic variants. (A–C) Comparison of serum FT3, FT4, and TSH levels at diagnosis between patients with *TSHR* biallelic variants and those with oligogenic variants including *TSHR*. (D–F) Comparison of serum FT3, FT4, and TSH levels at diagnosis between patients with *TSHR* biallelic variants and those with only the *TSHR* monoallelic variant. The Mann–Whitney U test was used to compare the serum FT4 and TSH levels between the two groups, and serum FT3 levels were compared using Student's *t*-test. *$P < 0.05$, **$P < 0.01$, ns, no significance.

Abbreviations: *TSHR*, thyroid-stimulating hormone receptor gene; FT3, free triiodothyronine; FT4, free thyroxine; TSH, thyroid-stimulating hormone.

REFERENCES

1. Yang RM, Zhan M, Zhou QY, Ye XP, Wu FY, Dong M, et al. Upregulation of GBP1 in thyroid primordium is required for developmental thyroid morphogenesis. Genet Med 2021;23:1944-51.

2. Kumar P, Henikoff S, Ng PC. Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. Nat Protoc 2009;4:1073-81.

3. Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, et al. A method and server for predicting damaging missense mutations. Nat Methods 2010;7:248-9.

4. Schwarz JM, Rödelsperger C, Schuelke M, Seelow D. MutationTaster evaluates disease-causing potential of sequence alterations. Nat Methods 2010;7:575-6.

5. Jagadeesh KA, Wenger AM, Berger MJ, Guturu H, Stenson PD, Cooper DN, et al. M-CAP eliminates a majority of variants of uncertain significance in clinical exomes at high sensitivity. Nat Genet 2016;48:1581-6.

6. Ioannidis NM, Rothstein JH, Pejaver V, Middha S, McDonnell SK, Baheti S, et al. REVEL: an Ensemble method for predicting the pathogenicity of rare missense variants. Am J Hum Genet 2016;99:877-85.

7. Jumper J, Evans R, Pritzel A, Green T, Figurnov M, Ronneberger O, et al. Highly accurate protein structure prediction with AlphaFold. Nature 2021;596:583-9.

8. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation

of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med 2015;17:405-24.

9. Abramowicz MJ, Duprez L, Parma J, Vassart G, Heinrichs C. Familial congenital hypothyroidism due to inactivating mutation of the thyrotropin receptor causing profound hypoplasia of the thyroid gland. J Clin Invest 1997;99:3018-24.

10. Zhang CX, Zhang JX, Yang L, Zhang CR, Cheng F, Zhang RJ, et al. Novel compound heterozygous pathogenic mutations of SLC5A5 in a Chinese patient with congenital hypothyroidism. Front Endocrinol (Lausanne) 2021;12:620117.

11. Brnich SE, Abou Tayoun AN, Couch FJ, Cutting GR, Greenblatt MS, Heinen CD, et al. Recommendations for application of the functional evidence PS3/BS3 criterion using the ACMG/AMP sequence variant interpretation framework. Genome Med 2019;12:3.