International Journal of General Medicine

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ORIGINAL RESEARCH

Variant of TSHR is Not a Frequent Cause of Congenital Hypothyroidism in Chinese Han Patients

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Purpose: To screen variants of the thyroid stimulating hormone receptor (*TSHR*) gene among congenital hypothyroidism (CH) patients.

Patients and Methods: We conducted a genetic screening of the *TSHR* gene in a cohort of 125 Chinese CH patients. Variants were detected by customized targeted next-generation sequencing.

Results: A total of 11 *TSHR* missense heterozygous variants were identified in 14 CH patients. Six variants were in the transmembrane domains, four variants were in the leucine-rich repeats and one variant was located in the hinge region of the TSHR protein. p.F525S was the most prevalent variant with an allele frequency of 0.016, followed by p.R450H with an allele frequency of 0.012. The allele frequency of most variants was higher in our cohort than those of other populations.

Conclusion: The prevalence of *TSHR* variants was 11.2%. Variant p.F525S was the most prevalent variant with an allele frequency of 0.016. The prevalence of *TSHR* variants was different from other populations.

Keywords: congenital hypothyroidism, thyroid stimulating hormone receptor, variant, prevalence

Introduction

Congenital hypothyroidism (CH) is the most common preventable cause of mental and motor retardation in infants with an incidence of 1:2000 to 1:4000.[1] With the development of molecular biotechnology, novel perspectives on the pathogenesis of CH have been reported. To date, numerous studies have reported genetic causes in CH patients, and several lines of evidence support a relevant genetic origin for CH.[2–5]

According to the causes of the underlying mutated genes, the genetic classification divides CH into two main categories, thyroid dysgenesis and thyroid dyshormonogenesis. The defects of thyroid dysgenesis are classified as agenesis (complete lack of thyroid tissue), ectopy (located in an improper position), hemiagenesis or hypoplasia (severely reduced thyroid size). Thyroid dysgenesis, which accounts for 80–85% of primary CH,[6] was reported to result from variants in genes responsible for the development or growth of the thyroid.[7] Variants in the paired box gene 8 (*PAX8*), thyroid transcription factor 1 (*TTF1*/*NKX2-1*) gene, thyroid transcription factor 2 (*TTF2/FOXE1*) gene and NK2 transcription factor related locus 5 (*NKX2-*

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Received: 31 May 2021
Accepted: 16 July 2021
Published: 3 August 2021

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5) gene were identified in patients with thyroid dysgenesis. Variants of the thyroid stimulating hormone (TSH) receptor (*TSHR*) gene have been shown to cause CH due to thyroid hypoplasia[8] or TSH resistance.[9] Variants in *THSR* result in a wide range of phenotypes ranging from severe CH to mild euthyroid hyperthyrotropinemia. Mild or severe hypothyroidism, thyroid in situ or apparent athyreosis, and complete or partial TSH resistance were both reported in patients with *TSHR* mutations.[10–12] In many previous studies, *TSHR* variants were reported mainly in patients with abnormal thyroid morphology because of the poor efficiency of the detection method. However, patients with normal thyroid morphology were not screened for variants in the *TSHR* gene.[12] With the use of next-generation sequencing, variants of *TSHR* were screened progressively in indiscriminate CH patients. The characteristics of *TSHR* variant have not been fully established, and little is known about the prevalence of variants among CH patients. More research is needed to clarify the causes and etiology of CH.

In the present study, we performed *TSHR* gene variant screening in a cohort of nonconsanguineous CH patients, aiming to screen and characterize variants in *TSHR*.

Materials and Methods

Patients

CH patients included in our study were identified from newborn screening between January 2010 and August 2019. Subclinical CH patients and patients with other congenital diseases were excluded from the present cohort. Informed consent to participate in this study was provided by the participants' legal guardians, and the study was conducted in accordance with the declaration of Helsinki. A total of 125 non-consanguineous Chinese Han patients were included in our study. The study design and protocol were reviewed and approved by the ethics committee of Changzhou Children's Hospital and the ethics committee of Changzhou Women and Children's Hospital affiliated to Nanjing Medical University.

CH Screening and Diagnosis

The flow path of screening and diagnosis of CH was based on the consensus statement of the Chinese Preventive Medicine Association.[13] Briefly, CH screening was performed between 72 h and 7 days after birth. Heel blood of neonates was dropped on filter paper, and dried blood spots were punched for the subsequent TSH test. TSH of newborns' heel blood (hTSH) was tested first by a time-resolved fluorescence assay. Newborns whose hTSH ranged from 9.0 to 20.0 mIU/L were recalled for a second test of hTSH. Newborns whose hTSH was higher than 20.0 mIU/L at the first test or whose hTSH was higher than 9.0 mIU/L at the second test were recalled again for the test of blood TSH and free thyroxine (FT4) to make a definite diagnosis. Serum TSH and FT4 were determined by electrochemiluminescence assay. The diagnosis of CH was based on elevated TSH levels and decreased FT4 levels. Thyroid morphology was determined using ultrasound scanning.

TSHR Variant Test

The *TSHR* targeted panel was designed based on the Illumina Sequencing Assay Designer, including entire coding regions and exon-intron boundaries of *TSHR* (chr14:81421965–81610778). Heel blood of patients was collected, and genomic DNA was extracted using the QIAGEN QIAamp DNA Blood Kit according to the manufacturer's protocol. Oligonucleotide probes were synthesized and pooled into a custom amplicon tube containing all the probes to generate attempted amplicons. Sample-specific indices were then added to each library by PCR using common primers from the TruSeq Amplicon Index Kit. After a normalization procedure enables simple volumetric pooling of libraries, sequencing was performed on the Illumina MiSeq 2000 system.

Variant Analysis

Illumina Amplicon Viewer was used for variant detection, data analysis, and variant annotation. The impact of variants on the function and structure of TSHR proteins was predicted by in silico tools, including SIFT, Polyphen-2 and MutationTaster. However, synonymous variants were not evaluated. Suspected pathogenic variants were also searched in public databases or previously published studies to interpret the pathogenic variants.

Results

Following the acquisition of consent from guardians, 125 newborns with CH (60 males and 65 females) were enrolled in our study. The average birth weight of the enrolled newborns was 3233 g, while the average gestational age was 38^{+5} weeks. The average level of hTSH in the newborns screening was 70.88 mIU/L, and

Table 1 Characteristics and Damage Prediction of Identified *TSHR* Variants in 14 Patients

Patient ID	Sex	hTSH (mIU/L)	TSH at Diagnosis (mIU/L)	FT4 at Diagnosis (pmol/L)	Thyroid Morphology	Variants	Location of Domain	SIFT	PolyPhen_2_HVAR	MutationTaster
1	M	69.5	>75	1.88	Normal	c.1574T>C, p.F525S	β, ICL 2	0.08	0.967	DC
2	M	15.2	>75	5.09	Undiagnosed	c.700T>C, p.S234P	α, LRR 8	0	0.998	DC
						c.1349G>A, p.R450H	β, ICL1	0	0.999	DC
3	M	19.5	>75	2.86	Normal	c.1349G>A, p.R450H	β, ICL1	0	0.999	DC
4	F	37.6	>75	7.13	Normal	c.1270G>T, p.V424F	β, TMD 1	0	1	DC
5	F	104	>75	1.98	Hypoplasia	c.1222T>C, p.C408R	α, Hinge region	0	0.999	DC
6	M	111	>75	3.35	Hypoplasia	c.1384T>C, p.C462R	β, TMD 2	0	0.999	DC
						c.1574T>C, p.F525S	β, ICL 2	0.08	0.967	DC
7	F	43	>75	6.1	Normal	c.823G>A, p.A275T	α, LRR 9	0.04	0.997	DC
8	M	10.5	>75	3.98	Ectopy	c.394G>C, p.G132R	α, LRR 4	0.14	0.784	DC
						c.1349G>A, p.R450H	β, ICL1	0	0.999	DC
9	F	9.34	59.49	5.99	Normal	c.1591C>T, p.R531W	β, ICL 2	0.01	0.998	DC
10	M	50	>75	1.23	Hypoplasia	c.394G>C, p.G132R	α, LRR 4	0.14	0.784	DC
11	M	141	>75	4.87	Goiter	c.1838A>G, p.Y613C	β, ICL 3	0.02	0.989	DC
12	M	15.5	>75	6.88	Normal	c.733G>A, p.G245S	α, LRR 8	0	0.998	DC
13	F	43.6	>75	5.54	Normal	c.1574T>C, p.F525S	β, ICL 2	0.08	0.967	DC
14	M	211	>75	1.01	Normal	c.1574T>C, p.F525S	β, ICL 2	0.08	0.967	DC

Notes: SIFT scores less than 0.05 are predicted to be deleterious, and those greater than or equal to 0.05 are predicted to be tolerated. Polyphen-2 score: "probably damaging" if the score is between 0.909 and 1, and "possibly damaging" if the score is between 0.447 and 0.908, and "benign" if the score is between 0 and 0.446.

Abbreviations: hTSH, heel blood TSH; M, male; F, female; TMD, transmembrane domain; ICL, intracellular loop; ECL, extracellular loop; LRR, leucine-rich repeat; DC, disease causing.

https://doi.org/10.2147/IJGM.S322726

the average levels of serum TSH and FT4 at diagnosis were 68.28 mIU/L and 3.97 pmol/L, respectively.

A total of 11 *TSHR* missense heterozygous variants were identified in 14 CH patients. The prevalence of *TSHR* variants was 11.2% in our unbiased cohort. Among these patients with *TSHR* variants, we observed the occurrence of thyroid dysgenesis (TD) in 6 patients and goiter in one patient, whereas 6 patients had normal-sized gland-in-situ (GIS), the characteristic of patients are shown in Table 1. Nine of 11 variants were included in the dbSNP (database of SNP) or gnomAD (Genome Aggregation Database, v2.1.1) databases, and two variants, p.S234P and p.C462R, were not included in any variation databases but were reported in previous studies. Of the 14 patients with mutated *TSHR*, 11 were single missense heterozygous. The remaining three patients harbored multisite heterozygous variants: p.S234P combined with p.R450H, p.C462R combined with p.F525S, and p.G132R combined with p.R450H, respectively.

Amino acids of six variants in the transmembrane domains (TMD) were located in the β subunit of the *TSHR* protein, including p.V424F, p.R450H, p.C462R, p.F525S, p.R531W and p.Y613C. Four variants were in the leucine-rich repeats (LRRs), including p.G132R, p.S234P, p.G245S and p.A275T. p.C408R was in the hinge region of the TSHR protein. The amino acid locations of the variants are shown in Table 1 and Figure 1.

The impact of variants on the function and structure of TSHR proteins was predicted by in silico tools. All

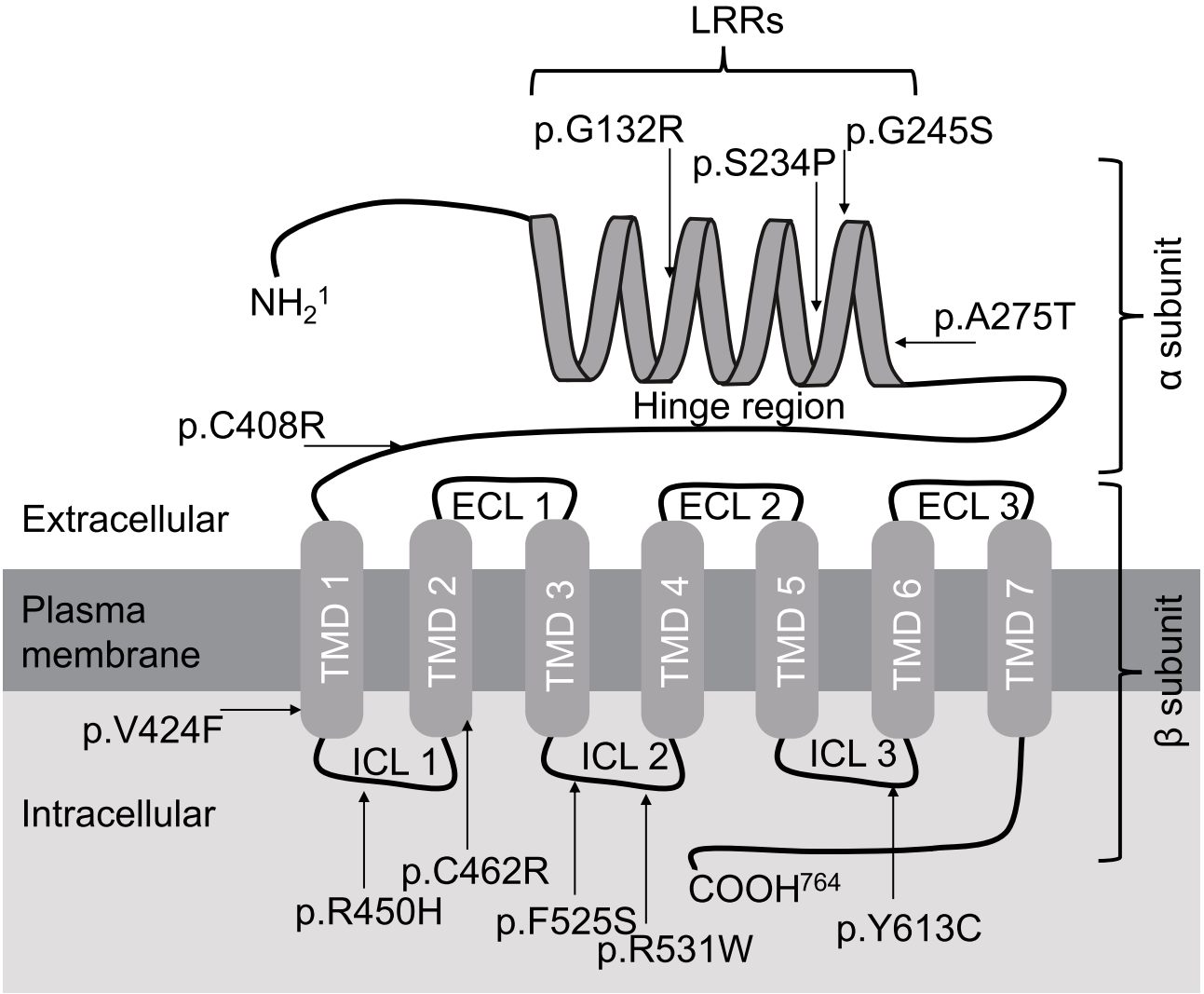

Figure 1 Model of *TSHR* protein structure and localization of variants that identified in the present cohort.
Abbreviations: TMD, transmembrane domain; ICL, intracellular loop; ECL, extracellular loop; LRR, leucine-rich repeat.

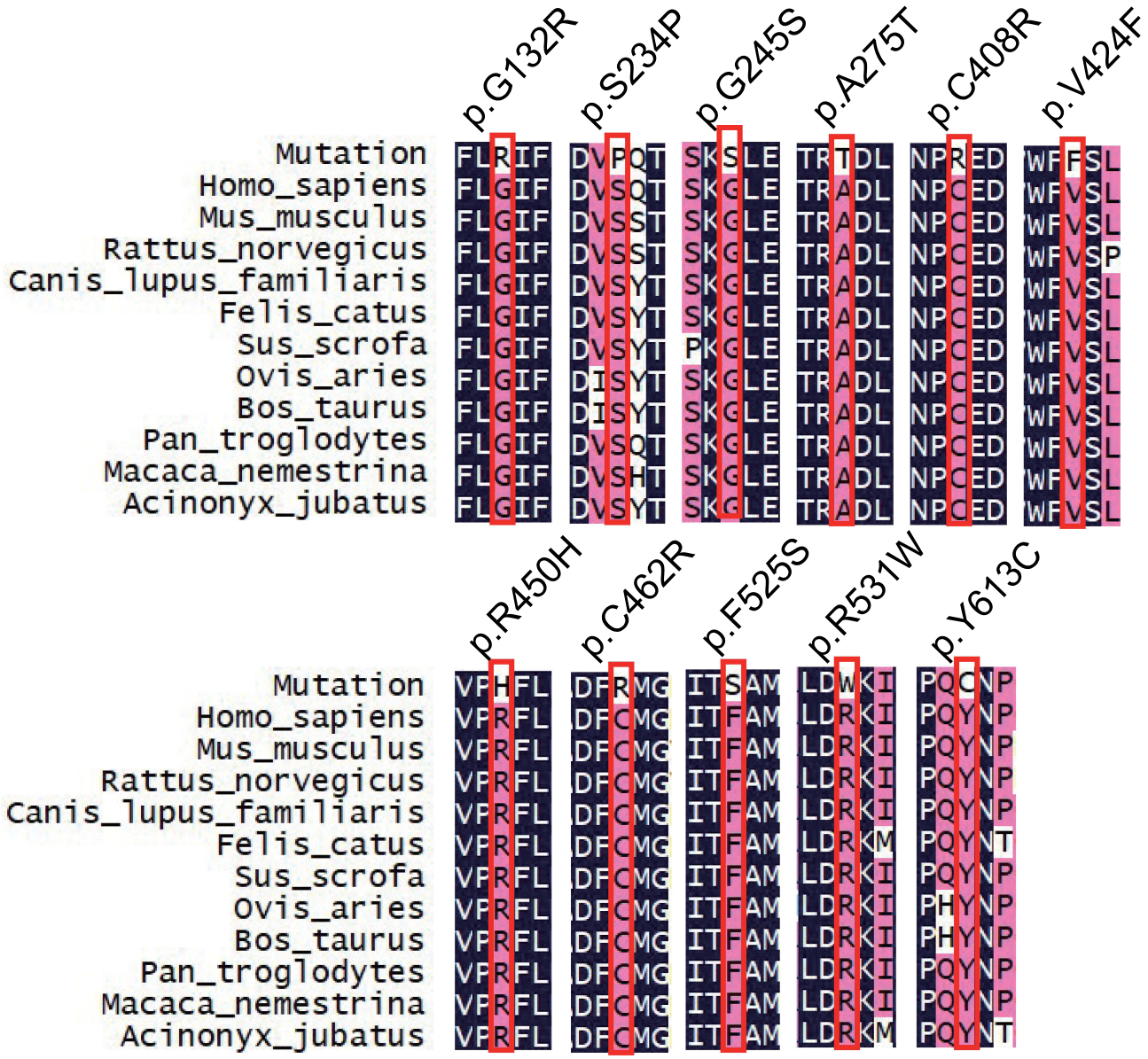

Figure 2 Multiple sequence alignment of *TSHR* protein among 11 different species.

variants were classified as "disease causing" by MutationTaster, nine of them were classified as "deleterious" by SIFT, and ten of them were classified as "probably damaging" by Polyphen-2 (Table 1). We also analyzed conservation by homology analysis among 11 different species, and most amino acid sequences of variants were located in the highly conserved regions of *TSHR* (Figure 2).

We reviewed the literatures and analyzed the various detection rate of *TSHR* variants among different populations in 23 studies,[10,14–35] the results are shown in Table 2. The prevalence of variants among different populations was also analyzed. In the present cohort, p.F525S was the most prevalent variant with an allele frequency of 0.016, subsequent p.R450H with an allele frequency of 0.012. The allele frequency of all nine variants was higher in our cohort than the allele frequency of the global population. However, the allele frequencies of six variants (p.G132R, p.C408R, p.V424F, p.R450H, p.F525S and p.R531W) were higher than those in East Asia (Table 3).

Discussion

In the present study, we studied *TSHR* variants by targeted sequencing. Targeted sequencing uses oligonucleotide probes designed to target and capture regions

Table 2 Detection Rate of T*SHR* Variants Among Different Populations

Nation	Patients	Detection Rate	Detection Method	Authors
China	CH	11.2% (14/125)	Target NGS	The present study
China	CH	1.67% (4/240)	Target NGS	Fu et al 2016[10]
China	Subclinical CH	4.17% (6/144)	Target NGS	Fu et al 2016[10]
China	CH	4.65% (2/43)	Target NGS	Wang et al 2020[14]
China	CH	7.27% (8/110)	Target NGS	Sun et al 2018[15]
China	CH	5.91% (13/220)	Target NGS	Fang et al 2019[16]
China	CH	1.52% (1/66)	Target NGS	Fan et al 2017[17]
China	CH	6% (6/100)	Target NGS	Wang et al 2017[18]
Japan	CH	3.68% (5/136)	Target NGS	Tanaka et al 2020[19]
Japan	CH	7.19% (12/167)	Target NGS	Yamaguchi et al 2020[20]
Japan	CH	5.88% (6/102)	PCR-based sequencing	Narumi et al 2009[21]
Japan	CH	12% (3/25)	Target NGS	Watanabe et al 2021[22]
Korea	CH	6.74% (13/193)	PCR-based sequencing	Lee et al 2011[23]
Korea	CH	5.29% (9/170)	PCR-based sequencing	Park et al 2016[24]
Korea	CH with GIS	30% (6/20)	Target NGS	Shin et al 2021[25]
Korea	CH with GIS	11.63% (5/43)	PCR-based sequencing	Jin et al 2014[26]
Italy	Subclinical hypothyroidism	28.95% (11/38)	PCR-based sequencing	Nicoletti et al 2009[27]
Italy	Subclinical hypothyroidism	11.9% (5/42)	PCR-based sequencing	Tonacchera et al 2004[28]
United Kingdom et al†	CH with GIS	2.5% (1/40)	NGS	Nicholas et al 2016[29]
Macedonia	CH	10% (4/40)	PCR-based sequencing	Zdraveska et al 2020[30]
Saudi Arabia	CH	10.91% (6/55)	NGS	Zou et al 2018[31]
Thailand	CH	4.24% (5/118)	Target NGS	Sorapipatcharoen et al 2020[32]
United Arab Emirates	CH	1.54% (1/65)	Target NGS	Deeb et al 2016[33]
Brazil	TD	0% (0/63)	PCR-based sequencing	Cerqueira et al 2018[34]
Hungary	PCH	4.71% (4/85)	PCR-based sequencing	Lábadi et al 2015[35]

Note: †: United Kingdom, Oman, Saudi Arabia, the United Arab Emirates, and Turkey.
Abbreviations: GIS, gland-in-situ; PCH, permanent CH; TD, thyroid dysgenesis; NGS, next generation sequencing.

of interest, followed by next-generation sequencing. This method enables researchers to analyze genetic variation in specific genomic regions. It also reduces sequencing costs and turnaround time compared to broader approaches such as whole-genome sequencing. In addition, ultradeep sequencing of PCR products allows efficient variant identification and characterization. Eleven missense variants were identified in a cohort of 125 patients with CH, and all the variants were heterozygous.

TSHR, which is located on the surface of thyroid follicular cells, is a member of the G-protein-coupled receptor superfamily. Variants in *TSHR* play a key role in the main regulatory cAMP and Gq/phospholipase C cascade pathways, which mediate most effects of hormone synthesis in the thyroid gland, including

Table 3 Allele Frequency of Identified Variants Among Different Populations

Variants	Allele Count	Allele Frequency			*P* †	*P* ‡
		Present Cohort	East Asian	Global		
p.G132R	2	0.008	0.0006	0.00004282	0.013	<0.001
p.G245S	1	0.004	0.0012	0.00008838	0.268	0.023
p.A275T	1	0.004	0.00033	0.00002386	0.09	0.007
p.C408R	1	0.004	0	0.000007953	0.013	0.003
p.V424F	1	0.004	0	0.00001768	0.012	0.005
p.R450H	3	0.012	0.00284	0.0002121	0.039	<0.001
p.F525S	4	0.016	0.00186	0.0001379	0.002	<0.001
p.R531W	1	0.004	0.00011	0.00002785	0.04	0.008
p.Y613C	1	0.004	0.00065	0.00004597	0.16	0.012

Notes: The data of allele frequency in East Asia and globally were quoted from gnomAD. *P* †: Comparison of allele frequency between the present cohort and East Asia. *P* ‡: Comparison of allele frequency between the present cohort and the global cohort.

iodide uptake, expression of thyroid genes, biosynthesis of thyroid hormone, TPO activity, thyroid H_2O_2 generating system, endocytosis, proteolysis and hormone release.[36] Loss-of-function variants in the *TSHR* gene are expected to cause uncompensated TSH resistance.[37,38] Most of these variants lead to misfolding of the protein, affecting the signaling pathway.[39] The TSHR ectodomain, consisting mainly of 9 LRRs and an N-terminal tail, forms the binding domain for TSH.[40,41] The 7 TMDs are joined intracellularly by connecting loops that interact with G proteins when the receptor is activated.[42] According to the function of the domains, variants in LRRs will result in decreased binding activity of TSH and subsequently lead to a reduction in cAMP production activities.[21,43] However, in vitro experiments confirmed that the TMD variant p.R450H not only leads to a reduction in cAMP production activities but also results in a decreased activity of TSH-binding.[21] Pathogenesis may be more complicated even contrary to the in silico analysis.[44]

A various prevalence of *TSHR* variants was reported among the different populations. The prevalence of *TSHR* variants was 11.2% in our cohort, which was higher than that in other Chinese population studies.[10,45] In contrast to the variants in *DUOX2* or *DUOXA2*,[17] *THSR* variants are not considered a major cause of CH in the Chinese population.[15] p.F525S was the most frequent variant in our cohort. Previous studies identified the variant among populations of Korea[23,46,47] and China,[48] indicating that p.F525S is a high allele frequency variant among East Asia. p.R450H was also identified as a high-frequency *THSR* variant in East Asia. It has been reported that p.R450H account for approximately 70% of *TSHR* variants in Japanese CH patients.[21] In Taiwanese CH patients, the frequency of homozygous p.R450H was 1.4% and that of heterozygous p.R450H was 5.6%.[49] p.R450H was the second most frequent variant, with a rate of 0.024 (3/125) in our cohort. While the variant rate of p.R450H was 0.0026 (1/384) in a previous study in Guangxi Zhuang Autonomous Region of China, and the rate was lower than that in our cohort. This demonstrated that the variant spectrum of *TSHR* is different among different populations. Other variants, p.G132R,[18] p.G245S,[23] p.V424F,[50] p.R531W[51] and p.Y613C,[10] were reported and are related to CH.

In the present cohort, the thyroid morphology was normal in most patients, although ectopy and hypoplasia were observed. It reported that phenotypic variability is a characteristic in patients with *TSHR* gene mutations, ranging from severe CH to only mild elevations of TSH in the absence of signs and symptoms of hypothyroidism.[52] In addition, the correlation between phenotype and genotype remains unclear. Previous studies indicated that heterozygous *TSHR* mutations have been associated with mildly elevated TSH levels, and biallelic mutations in the *TSHR* gene result in mild or moderate hypothyroidism with high TSH concentrations, or severe hypothyroidism with a hypoplastic thyroid gland or athyreosis.[53] However, studies have also reported that heterozygous mutations were identified in patients with athyreosis,[18] and the monoallelic *TSHR* mutations were recognized as

a pathologic role of CH.[54] Further studies are required to clarify the molecular etiology and genotype-phenotype correlation in CH with *TSHR* mutations.

Conclusion

In the present study, we conducted a variant screening of the *TSHR* gene by customized targeted next-generation sequencing. The prevalence of *TSHR* variants was 11.2% in our cohort, and the prevalence of *TSHR* variants was different from other populations. The identification of variants could contribute to the accurate diagnosis and classification of defects, and it also helps to further understand the gene variant spectrum and genetic pathogenesis of CH.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (grant number 81903400), Changzhou Key Laboratory of High-tech Research (grant number CM20193009), Changzhou Social Development Science and Technology Support Project (grant number CE20205035), Young Talent Development Plan of Changzhou Health Commission (grant number CZQM2020096).

Disclosure

The authors report no conflicts of interest in this work.

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