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Original Article

Identification of a novel *TSHR* mutation from a Chinese baby with congenital hypothyroidism due to ectopy

Li Li[1*], Wenhui Zhang[1,2*], Yucui Zang[3,4], Shengli Yan[1], Bin Kong[5], Shiguo Liu[3,4]

[1]Department of Endocrinology, [3]Center of Prenatal Diagnosis, [4]Genetic Laboratory, [5]Department of Breast Surgery, The Affiliated Hospital of Qingdao University, Qingdao, China; [2]Department of Endocrinology, Liaocheng People's Hospital, Liaocheng, China. [*]Equal contributors.

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Abstract: Background: Congenital hypothyroidism (CH) is characterized by elevated levels of TSH with simultaneous low serum T4 and T3 levels because of reduced thyroid function. About 85% of CH cases are associated with thyroid dysgenesis, but its pathogenesis remains unclear. Aim: To screen *TSHR* mutations in Chinese CH patients with ectopy. Materials and methods: After collecting blood samples from 89 CH babies with ectopy, genomic DNA was extracted and exon 10 in *TSHR* mutations were detected by PCR as well as direct sequencing. Results: A novel missense mutation c.1270G>A, which results in the substitution of a valine at position 424 by an isoleucine residue (p.Val424Ile) was found according to direct sequencing of 89 subjects. Conclusion: We report a novel heterozygous missense mutation in one out of 89 unrelated Chinese CH patients with ectopy.

Keywords: Congenital hypothyroidism, ectopy, thyrotropin receptor, mutation

Introduction

Congenital hypothyroidism (CH) is one of the most common neonatal endocrine disorders, presenting with abnormal growth and intellectual impairment due to loss of thyroid function at different levels. It occurs at an incidence of 1 in 3000 to 4000 infants [1] and affects twice as many females as males [2]. Without thyroid hormone replacement promptly, the physical and mental disability can be permanent. Clinical manifestations of CH mainly include poor feeding, prolonged jaundice, edematous, umbilical hernia and dry skin. All the CH patients can be divided into 2 groups: about 85% of cases are caused by thyroid dysgenesis, including agenesis (22-42%), ectopy (35-42%), and hypoplasia (24-36%) [3], whereas the left 15% are associated with dyshormonogenesis. Many researchers suggested that thyrotropin receptor (*TSHR*) [4], paired box transcription factor 8 (PAX8) [5], NK2 homeobox 1 (NKX2-1) [6] and Forkhead box E2 (FOXE2) [7] play important roles in the differentiation and growth of the embryonic thyroid gland and are essential for normal thyroid development. At the same time, a variety of candidate genes mutations have been proven to affect the thyroid hormone synthesis, such as thyroglobulin (TG) [8], thyroperoxidase (TPO) [9], odium/iodide symporter (NIS) [10], dual oxidase 2 (DUOX2) [11], DUOX maturation factor 2 (DUOXA2) [12], pendrin (PDS) [13], and iodotyrosine deiodinase (DEHAL1) [14].

The human *TSHR* is localized on chromosome 14q31, and encodes for a G-protein-coupled receptor with a classical seven transmembrane domain (TMD) interacting with G proteins and an unusually large (350-400 aa) extracellular domain (ECD) responsible for high-affinity hormone binding [15]. *TSHR* ECD is encoded by the first 9 exons and part of exon 10, whereas the TMD and intracellular domain are encoded entirely by the exon 10 [16]. The main function of *TSHR* is to generate cAMP as second messenger according to the combination with TSH, which play a series of biological effects in thyroid organogenesis and development. *TSHR* mRNA is detected in the developing thyroid after the completion of the migration of the primordium before the first evidence of follicular

Table 1. *TSHR* exon10 oligonucleotides used as primers in PCR amplification

Nucleotide sequences			Fragment length (bp)
10-1	Forward primer	GCCTGGCACTGACTCTTT	447
	Reverse primer	AGTTTGTAGTGGCTGGTGA	
10-2	Forward primer	TCACCAGCCACTACAAACT	446
	Reverse primer	GATGACGAAGGCAACTATG	
10-3	Forward primer	AGTCCGAAATCCGCAGTA	561
	Reverse primer	AGAGTGAGGGCAGCTATG	

organization in the gland. The activation of *TSHR* regulates both proliferation and functioning of adult thyroid cells.

The analysis of thyroid development in mice carrying spontaneous or induced alterations in *Tshr* has provided a powerful tool in the exploration of the role of the TSH/Tshr pathway during embryonic life [17].

So far many *TSHR* inactivating mutations have been identified in cases of CH. Most of these mutations are located in exon 10 of *TSHR*, which indicates that exon 10 is mutation high-risk regions in TSHR [18]. The mutational spectrum of *TSHR* and the genotype-phenotype relationships have not yet been fully established. Here, we screened exon 10 of *TSHR* mutations in CH patients with ectopy to characterize the features of *TSHR* mutations in China.

Materials and methods

Patients

A total of 89 CH patients with ectopy (27 boys, 62 girls, age 5.2±1.6 years) were recruited through the neonatal screening program in Qingdao, Yantai, Weifang, and Linyi in Shandong Province, China, from 2008 to 2013. All of the subjects enrolled in the study came from iodide-sufficient areas, without any other congenital diseases. According to neonatal screening using filter paper for CH between 72 h and 7 days after their full-term or premature birth, all measurements were done using the same assay at four different laboratories in the relevant cities. All of the subjects underwent neonatal screening using filter paper for CH at 72 hours after birth; 0.8 ml blood samples were collected from the heel and TSH level was measured by enzyme-linked immunosorbent assay (ELISA). Subjects with increased TSH (TSH ≥20 uIU/ml) levels observed during neonatal screening were recalled for further evaluation. Serum TSH (normal range 0.27-4.2 uIU/ml), free thyroxin (FT4, normal range 12-22 pmol/L) were determined by electrochemiluminescence assay. CH was diagnosed with an elevated thyroid stimulating hormone (TSH) and a low free thyroxin (FT4) level. Typical clinical symptoms, including prolonged jaundice, constipation, poor feeding, umbilicalhernia, macroglossia, wide open posterior fontanel and edematous and dry skin [19] and ultrasonic examination or 99mTc thyroid scan confirmed the diagnosis. Blood samples were collected after written informed consent from parents or responsible was obtained and the research project was approved by the Ethics Committee of the Affiliated Hospital of Qingdao University (2013-qyfy22).

DNA analysis

Genomic DNA was extracted from peripheral blood leukocytes using the phenol-chloroform extraction method. The exon 10 in *TSHR* (NM_000369.2) was amplified by polymerase chain reaction (PCR) according to primers as follows (**Table 1**). The mutations in all *TSHR* coding regions would be screened if the subjects were identified with *TSHR* mutation in exon 10. Identical amplification conditions were used in a total volume of 25 μl containing 250 nM dNTPs, 100 ng of template DNA, 0.5 μM of each primer, and 1.25 U AmpliTaq Gold DNA polymerase, in 1× reaction buffer (10 mM Tris HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$). The samples were denatured at 94°C for 5 min followed by 35 cycles of amplification consisting of 30 s 94°C, 1 min 51-65°C and 30 s 72°C, and a final primer extension of 10 min 72°C. Amplified PCR products were purified and sequenced using the appropriate PCR primers and the BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA), and run on an automated sequencer, ABI 3730XL (Applied Biosystems, Foster City, CA, USA), to perform mutational analysis.

SIFT software (Sorting Intolerant from Tolerant, http://sift.jcvi.org) was used to predict tolerance to mutations on the basis of sequence conservation in the protein family and the PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2) was used to predict the influence of mutations based on data derived from struc-

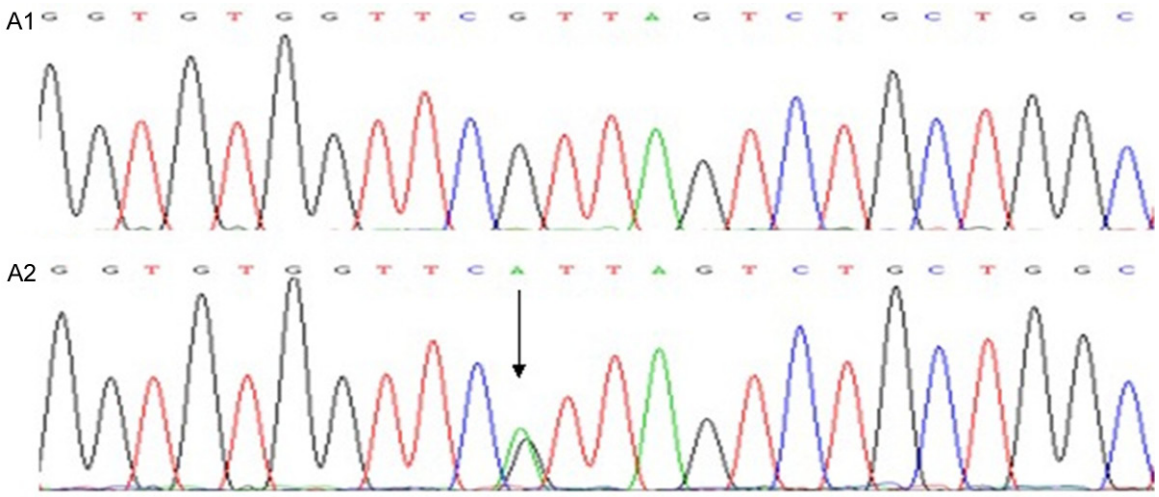

Figure 1. Partial sequences of exon 10 in TSHR from normal and affected individuals are shown. Arrow indicates the heterozygous G and A at nucleotide 1269 in the affected patient.

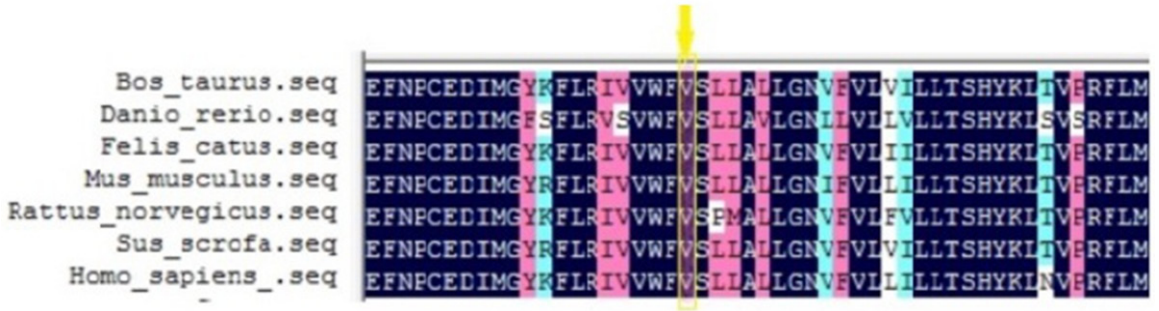

Figure 2. Multiple sequence alignment of TSHR from Homo sapiens, Mus musculus, Rattus norvegicus, Danio rerio, Felis catus, Sus scrofa and Bos Taurus. The valine 424 residue is located within a highly conserved region.

tural parameters, functional annotations and evolutionary information.

Results

Mutation analysis of TSHR

Analysis of the *TSHR* of the patients revealed a novel missense mutation c.1270G>A, which was predicted to result in a valine to isoleucine substitution at codon 424 in exon 10 (p.Val424Ile) (**Figure 1**). The mutation was not found in 100 control individuals and the age range of the controls is comparable with the age range of the patients. SIFT software was used to predict tolerance to this missense mutation (SIFT score 0) and the PolyPhen-2 was used to predict the influence of this mutation (PolyPhen-2 score 0.979). From the NCBI website, we obtained the TSHR family protein-sequence of various species, including Homo sapiens, Mus musculus, Rattus norvegicus, Danio rerio, Felis catus, Sus scrofa and Bos Taurus. Using DNAMAN software (Lynnonon Biosoft, Quebec, Canada), we achieved multiple sequence alignment of the TSHR family of different species. We found that codon 424 where the mutation p.Val424Ile was identified, was located in highly conserved region of TSHR (**Figure 2**). In addition, *TSHR* mutation screening in other coding regions was negative in this subject. Because of a lack of data, we were unable to perform mutation segregation with phenotype within the family.

Clinical data about the patient

The patient carrying c.1270G>A mutation in TSHR, is a male subject. His birth height/weight were 50 cm/2850 g. He was diagnosed with CH via the neonatal screening program, and the initial TSH level obtained from a dried blood spot was 146.7 uIU/ml (upper limit, 20 uIU/ml). He was recalled for further evaluation at the

age of 21 days. The TSH level was 194 uIU/ml and the FT4 level was 4.0 pmol/L.99 mTc thyroid scan showed an ectopic thyroid. Euthyrox replacement (8.1 μg/kg/d) was started immediately after the diagnosis of CH. Now at the age of 2.5 years, under replacement therapy of 50 ug/d L-T4, he was 89 cm tall and weighed 12 kg with normal intellectual development.

Discussion

Thyroid development begins as a cell condensation and evagination in the anterior embryonic region and proceeds by proliferation, migration and bifurcation with expression of TTF-1, TTF-2 and PAX-8. When at day 15 of mice and week 10 of human development, TSHR, as a thyroid specific proteins, occures to expression and complets the process of development and leading to maturation of the gland by follicular growth. Due to the time of its expression and the suspected functional role during follicle maturation, a functional defect in *TSHR* would not interfere with early stages of organogenesis but with thyroid growth during further maturation. Therefore, *TSHR* mutations may result in a phenotype with dysgenesis including ectopy. *TSHR* mutation is divided into activating mutation and inactivating mutation. Activating mutations causing constitutive activation of TSH receptor have been described in patients with familial and sporadic forms of non autoimmune hyperthyroidism and in autonomous thyroid nodules [20]. Inactivating mutations are, instead, the most frequent cause of TSH resistance. In some cases, this resistance causes CH. Evidence of *TSHR* inactivating mutations causing CH was firstly found in study of the congenital hypothyroid (hyt/hyt) mouse, which has been described as having a homozygous recessive mutation of a single locus on chromosome 12 which results in significant endocrine hypofunction and retarded growth [21]. The subsequent finding that in *TSHR* hyt/hyt mice a homozygous loss-of-function mutation in the *TSHR* impairs the binding of TSH has validated the hypothesis that *TSHR* is a candidate gene for CH with dysgenesis.

Since the first family with a documented *TSHR* inactivating gene mutation were reported in 1995 [22], at least 47 mutations have been described so far, nearly spreading over all the exons and including 33 missense mutations, 6 nonsense mutations, 6 frame shifts and 2 intronic mutations. Nevertheless, some mutations such as p.C41S, p.P162A, p.C390W, p.R450H, p.W546X and p.A553T have been reported more than once [23-25]. In particular, the mutations p.W546X and p.R450H appear to be major contributors to thyroid dysfunction in the Welsh and Japanese populations respectively [23, 24]. Most of these mutations are located in exon 10, so our study focused on screening mutations in exon 10 of *TSHR* in 89 Chinese CH patients with ectopy.

Inactivating mutations were first described in three sisters of a family with normal levels of circulating T3 and T4, but chronically elevated plasma TSH [22]. The trait appeared to be transmitted in the autosomal recessive mode, as thyroid tests were normal in both heterozygous parents, except for a slightly elevated TSH. All three were compound heterozygotes for missense mutations affecting closely located amino acids in the extracellular domain of the receptor. The p.P162A mutation (inherited from the mother) displayed some residual activity when expressed transiently in COS-7 cells, whereas the paternal mutation p.I167N displayed no detectable residual activity. In 1996, de Roux N [25] observed four families with loss of function mutations of the *TSHR*. One patient had a homozygous P162A substitution, the three other were compound heterozygotes: Q324X/D410N, C41S/F525L, C390W/W546X. In all patients, the plasma TSH concentration was increased, whereas T3 and T4 concentrations were normal. Both p.Q324X and p.W546X were novel nonsense mutations, and expressions of the mutated receptors in transfected COS-7 cells demonstrated the impairment of their function, including the reduced expression of the receptors on the cell surface by immunofluorescence, the declined ability to bind hormone and activate adenylate cyclase.

With increasing mutations identified, the genotype-phenotype and structure-function correlations of the *TSHR* turned more complicated. Jeziorowska A [26] reported a homozygous nonsense mutation, p.Y444X, in the first intracellular loop of *TSHR*, rendering a truncated receptor. Thus, the observed unresponsiveness to *TSHR* may be due to absent insertion of the truncated receptor into the cell membrane (if it gets translated at all) or the truncation may lead to nonsense-mediated mRNA degradation (its unresponsive to TSH). Tonacchera M [27] identified *TSHR* mutations in seven members

of three families with subclinical hypothyroidism, and discovered 41TGCAins resulted in a premature stop of translation at codon 62 (p.Q8fsX62). The proband was heterozygous for the mutation p.Q8fsX62. After transfection in COS-7 cells, the mutant receptor Q8fsX62 displayed a low expression at the cell surface, and a reduced response to bovine TSH (bTSH) in terms of cAMP production.

The novel *TSHR* mutation in exon 10 identified in this present study is located within the first transmembrane helix of *TSHR*. Based on reported crystal structure of *TSHR*, p.V424I mutation is located in highly conserved region of *TSHR*, consequently may influence the functional properties of the protein by changing its transactivation ability. The patient harboring p.Val424Ile mutation suffered from severe CH with obvious thyroid hypoplasia. Although *TSHR* mutation can cause a genetic autosomal recessive disorder characterized by the presence of inactivating gene variants in both alleles, only one variant has been found in exon 10 after analysis of the other whole coding sequence of *TSHR* in the present case. The possible reasons may be that another mutation may exist in intron or regulatory region of *TSHR*.

Here, we analyzed exon 10 of *TSHR* of 89 unrelated CH patients with ectopy in China by direct sequencing and identified a novel mutation (c.1270G>A/p.Val424Ile) in a CH patients. Even if bioinformatics tools such as PolyPhen-2 and SIFT predict this variant as a probably pathogenic one, in vitro expression studies are required to confirm the effect of this variant on receptor function in the future.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Bin Kong, The Affiliated Hospital of Qingdao University, 16 Jiangsu Road, Qingdao 266003, China. E-mail: qkongbin@163.com; Dr. Shiguo Liu, Center of Prenatal Diagnosis, The Affiliated Hospital of Qingdao University, Qingdao 266003, China. E-mail: liushiguo2002@126.com

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