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

Li Li1*, Wenhui Zhang1,2*, Yucui Zang3,4, Shengli Yan1, Bin Kong5, Shiguo Liu3,4

1Department of Endocrinology, 3Center of Prenatal Diagnosis, 4Genetic Laboratory, 5Department of Breast Surgery, The Affiliated Hospital of Qingdao University, Qingdao, China; 2Department 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 (NKK2-1) [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], thyperoxidase (TPO) [9], odium/iodide symporter (NIS) [10], dual oxidase 2 (DUOX2) [11], DUOX maturation factor 2 (DUOX2) [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...
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The activation of TSHR regulates both proliferation and function-ing 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 ulU/ml) levels observed during neonatal screening were recalled for further evaluation. Serum TSH (normal range 0.27-4.2 ulU/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, umbilical hernia, macroglossia, wide open posterior fontanel and edematous and dry skin [19] and ultrasonographic 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 MgCl2). 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-

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

<table>
<thead>
<tr>
<th>Nucleotide sequences</th>
<th>Fragment length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-1 Forward primer</td>
<td>GCCTGGCAGTCTTT</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>AGTTGATGGCTGGTGA</td>
</tr>
<tr>
<td>10-2 Forward primer</td>
<td>TCACCAGCCACTAAACT</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>GATGACGAGGCAACTAG</td>
</tr>
<tr>
<td>10-3 Forward primer</td>
<td>AGTCGAAATCCGCAGTA</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>AGAGTGAGGGCAGCTATG</td>
</tr>
</tbody>
</table>
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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 Bioskf, 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
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age of 21 days. The TSH level was 194 ulu/ml and the FT4 level was 4.0 pmol/L. 99 mCi thyroid scan showed an ectopic thyroid. Euthyroid 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, occurs to expression and completes 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 in-frame 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: liushigu2002@126.com

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