Mutation screening of the TPO gene in a cohort of 192 Chinese patients with congenital hypothyroidism

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ABSTRACT

Objectives: Defects in the human thyroid peroxidase (TPO) gene are reported to be one of the causes of congenital hypothyroidism (CH) due to dysmorphogenesis. The aim of this study was to examine the TPO mutation spectrum and prevalence among patients with CH in the Guangxi Zhuang Autonomous Region of China and to define the relationships between TPO genotypes and clinical phenotypes.

Methods: Blood samples were collected from 192 patients with CH in the Guangxi Zhuang Autonomous Region, China and genomic DNA was extracted from peripheral blood leukocytes. All exons of the 10 common CH-associated genes including TPO together with their exon-intron boundaries were screened by next-generation sequencing (NGS). The effect of the novel TPO mutation was investigated by ‘in silico’ studies.

Results: NGS analysis of TPO in 192 patients with CH revealed 3 different variations in 2 individuals (2/192, 1%). Sequencing other CH candidate genes in the patients with TPO variants revealed that patient 1 was homozygous for c.2422delT TPO mutation combined with double heterozygous DUOX2 pathogenic variants (p.R683L/p.L1343F) and patient 2 was trisomic for TPO pathogenic variants (p.R648Q/p.T561M/p.T561M). The present study identified a novel TPO variation c.1682C>T/p.T561M; and four known mutations: c.2422delT/p.C808Afs×24 and c.1943C>T/p.T561M). The present study identified a novel TPO mutation investigated by ‘in silico’ studies.

Conclusions: Our study indicated that the prevalence of TPO mutations was 1% among studied Chinese patients with CH. More than two variations in one or more CH-associated genes can be found in a single patient, and the coexistence of multiple mutations may exacerbate the severity of the hypothyroid condition.

Strengths and limitations of this study

- We conducted one of the largest TPO mutation screenings, in a cohort of 192 patients with congenital hypothyroidism (CH), in the Guangxi Zhuang Autonomous Region, China.
- We identified that the prevalence of TPO pathogenic variants was 1% among patients with CH.
- More than two variations in one or more CH-associated gene can be found in a single patient, and the coexistence of multiple mutations may exacerbate the severity of the hypothyroid condition.
- We found a novel TPO variation, thereby expanding the mutational spectrum of the gene.
- The relationships between TPO genotypes and clinical phenotypes could not be clearly determined because only two patients were identified to be mutation-positive participants and both had multiple variations.

INTRODUCTION

Congenital hypothyroidism (CH) is a common endocrine disorder with prevalence ranging from 1:2000 to 1:4000 newborns. Apart from iodine deficiency, which is still a major cause for endemic hypothyroidism in neonates, the sporadic CH cases can be classified into two groups: (1) disorders of thyroid gland development (thyroid dysgenesis) that have been linked to mutations in TSHR, PAX8, NKX2.1 and FOXE1 genes, which account for the majority of cases (80–85%); and (2) abnormalities in thyroid hormone synthesis (dysmorphogenesis), which account for the remaining 15–20% of cases and are associated with mutations in DUOX2, TG, TPO, SLC5A5, SLC26A4 and IYD genes.

Defects in the human TPO (NM_000547) gene are reported to comprise some of the most common causes of thyroid dysmorphogenesis (TDH). TPO, a thyroid-specific haeme peroxidase localised in the apical membrane of thyrocytes, plays a vital role in thyroid hormone biosynthesis. CH caused by mutations in the TPO is an autosomal
recessive disorder, and most patients with biallelic TPO mutations have permanent congenital hypothyroidism (PCH). Up to now, about 100 mutations have been reported and recorded in the HGMD (http://www.hgmd.cf.ac.uk/ac/index.php), but the genotype-phenotype relationship has not yet been fully established, and little is known about its mutational spectrum and prevalence among Chinese patients with CH. We performed the TPO gene screening in a cohort of 192 patients with CH in Guangxi Zhuang Autonomous Region, China.

MATERIALS AND METHODS

Patients

We enrolled 192 patients (101 females and 91 males) with CH; the previous 45 patients with CH collected for DUOX2 mutation screening were not included in this study.11 Most patients were initially identified by neonate screening among 623,000 newborns in the Guangxi Zhuang Autonomous Region, China, from October 2009 to June 2014. The thyroid gland was normal in size and position in 75 of these patients; 72 patients had increased and 47 showed decreased size of the thyroid gland. Newborn screening was performed with filter paper for CH between 72 h and 7 days after birth. Blood samples were collected from the heel and the thyroid-stimulating hormone (TSH) level was measured by time-resolved fluorescence assay (Perkin Elmer, USA). Newborns with increased TSH (TSH ≥8 mIU/L) levels observed during neonatal screening were followed-up for further evaluation. Serum TSH and FT4 were determined by electrochemiluminescence assay (Cobas e601, Roche Diagnostics, USA). Diagnosis of CH was based on elevated TSH levels (TSH >10 mIU/L) and decreased FT4 levels (FT4 <12 pmol/L). Permanent or transient CH was determined using results of thyroid function tests after temporary withdrawal of L-T4 therapy at 2 years of age. The perchlorate discharge test, which aids in the recognition of iodide organification defects, is not routinely performed. The study was approved by the Medical Ethics Committee of Guangxi Maternal and Child Health Hospital. Informed consent was obtained from the parents of the patients.

Mutation detection and interpretation

Peripheral venous blood samples were collected from the patients. Genomic DNA was extracted from peripheral blood leukocytes, using QIAamp DNA Blood Mini Kit (Qiagen, Germany) according to the manufacturer’s protocol. A CH capture panel an Illumina Truseq Custom Amplicon V1.5 kit, included 10 known CH-associated genes (TPO, THSR, PAX8, NKX2.1, FOXE1, DUOX2, TG, SLC5A5, SLC26A4 and IYD) with complete coding regions and flanking intronic regions; the DUOX2 gene was not included. The prepared sample library was sequenced employing an Illumina MiSeq instrument using MiSeq Reagent Kit V2, 500-cycles (Illumina Inc, San Diego, California, USA). Illumina Amplier Viewer V1.3 and MiSeq Reporter V2.3 software were used for data analysis, and the SnpEff12 was used for variant annotation. Polyphen 213 and MutationTaster14 were used to evaluate the pathogenicity of the novel mutations. DNAMAN software V8 was then used to carry out multiple sequence alignment of the TPO family protein from different species. In addition, a cohort of 300 ethnicity-matched healthy participants was used to assess the variant frequencies in the normal control. All control participants had normal FT4 and TSH levels.

Sanger sequencing

Sanger sequencing was used to validate the mutations identified from next-generation sequencing.

In silico studies of the novel TPO gene mutation

The crystal structure of human TPO is currently not available. To study the three-dimensional (3D) structure of the p.T561M TPO, we modelled the wild-type and mutant TPO variants in silico, using the Swiss Model program package.15,16 Multiple templates were used to generate different target models, and the one presenting the best sequence identity percentage was chosen. Thus, a homology model was generated with human myeloperoxidase isofrom C (PDB accession number 1mhl) as a template. Myeloperoxidase showed an overall identity of 48.16% for amino acids 258-735 of TPO. Verify3D software was used to determine the compatibility of the 3D atomic model with its own 1D amino acid sequence. The structure with lowest potential energy was extracted, verified using Ramachandran plots that were generated by Procheck software (http://nihserver.mbi.ucla.edu/SAVES), to check the stereo chemical quality of the protein. We then submitted the chosen model to the Swiss PDB Viewer17 to determine potential structural differences between the mutant and the wildtype TPO.

RESULTS

Analysis of TPO and other CH-associated genes by next-generation sequencing

PCR-based targeted enrichment, using Illumina Truseq Custom Amplicon, of 10 known CH genes followed by 500 cycles paired-end sequencing on an Illumina MiSeq was performed, resulting in a total of 4.77 million reads. The average coverage is more than 95%; all TPO exon depths were successfully covered with >40×. Also, no deletion or duplication of exons in TPO was detected by manual read depth inspection. In this study, we identified three different TPO variants in two individuals. Sequencing of other CH candidate genes in the two patients with the TPO mutations showed that patient 1 was homozygous for c.2422delT TPO mutation combined with double heterozygous DUOX2 mutations (p.R683L/p.L1343F), and patient 2 was triallelic with TPO pathogenic variants (p.R648Q/p.T561M/p.T561M). All variants were confirmed by Sanger sequencing (figures 1, 2).
and 2). The present study identified a novel variation: p.\(T561M\) in the \(TPO\); and four known mutations: c.2422delT and p.R648Q in \(TPO\), p.R683L and p.L1343F in \(DUOX2\). Polyphen2 and Mutation taster predicted that the novel variation p.\(T561M\) would have deleterious effects by damaging the \(TPO\) protein. Additionally, the variant was not detected in our normal control population. DNAMAN software was then used to carry out multiple sequence alignment of the \(TPO\) family protein from different species; the identified variation was found to be located in the highly conserved region of \(TPO\) (figure 3). These all suggested that the amino acid substitution might be due to a pathological mutation.

**In Silico predictions of functional impact of the T561M on the TPO protein**

The generated 3D homology models based on 1mhl template (44% sequence similarity) covered 50% of the full sequence of \(TPO\) protein. Verify3D showed that the model had passed as a good model by having 86.6% of residues with an average 3D to 1D score of more than 0.2. Ramachandran plots from Procheck showed that the model had three amino acid (Gly331, Glu384 and Lys627) residues located in the disallowed regions. However, these amino acid residues were distant from the active site. Consequently, the homology model was acceptable for subsequent analyses.

The threonine 561 residue is located within a highly conserved region of \(TPO\), which suggests an important role in the function and/or structure of \(TPO\). Comparison between predicted tertiary structures of the wild type and mutant proteins revealed that the substitution, p.\(T561M\), breaks two hydrogen bonds with an asparagine in position 557 and another hydrogen bond with a glutamic acid in position 558. Significant structural alterations in the annotation were also detected in the p.\(Thr561Met\) mutant (figure 4). As a result, these changes will disturb the secondary structure and affect the function of \(TPO\) protein.

**Clinical features and laboratory test results of the patients**

The clinical features and laboratory results are summarised in table 1. Patient 1 was born at full-term to non-consanguineous parents. His birth weight was 3000 g and length 47 cm. There was no family history of thyroid disease. Newborn screening at the age of 6 days...
revealed a high level of TSH (78.6 mIU/L). He was therefore re-evaluated at hospital at the age of 13 days. A serum TSH level of >100 mIU/L (normal range 0.7–10 mIU/L), FT4 level of 1.92 pmol/L (normal range 12–32 pmol/L) and FT3 level of 1.68 pmol/L (normal range 2.65–9.68 pmol/L) confirmed the diagnosis. Ultrasound examination showed an enlarged thyroid gland (right lobe 3.4×1.5×1.4 cm; left lobe 3.2×1.4×1.3 cm). L-T4 replacement therapy was started immediately at an initial daily dose of 12 μg/kg and adjusted according to the serum TSH, FT4 and FT3 levels. The diagnosis of PCH was confirmed due to a high TSH level (38.87 mIU/L) after temporary withdrawal of L-T4 therapy for 4 weeks at the age of 2.3 years. The L-T4 replacement therapy was then resumed. The patient is now 3.1 years of age and receives a daily dose of 6 μg/kg L-T4. His physical and intellectual development is appropriate to his age. Family studies showed that his father carried a heterozygous TPO mutation c.2242delT and a heterozygous DUOX2 mutation p. R683L but with no thyroid phenotype, and his mother harboured the heterozygous TPO mutation c.2242delT without abnormal thyroid phenotypes (figure 1).

Patient 2 was diagnosed with CH in our outpatient paediatric endocrinology clinic. The boy was 4.5 years old at the time; he was 98 cm (<3rd) in height and 13.5 kg (<3rd) in weight; he had a TSH level of >100 mIU/L (normal range 0.7–10 mIU/L), and his FT3 and FT4 were 4.2 pmol/L (normal range 2.65–9.68 pmol/L) and 0.3 pmol/L (normal range 12–32 pmol/L), respectively. Thyroid ultrasound showed evidence of goitre (right lobe 3.0×1.3×1.2 cm; left lobe 3.2×1.6×1.4 cm). L-T4 replacement therapy was started immediately at an initial daily dose of 15 μg/kg and adjusted according to the serum TSH, FT4 and FT3 levels. The diagnosis of PCH was confirmed due to a high TSH level (40.6 mIU/L) after temporary withdrawal of L-T4 therapy for 5 weeks at the age of 6.7 years. At his last visit to our hospital, the patient was 7 years of age and was receiving a daily dose of 6 μg/kg L-T4. His weight was 17.5 kg (<3rd) and height 113 cm (<3rd). Physical examination and IQ testing showed physical and mental retardation compared to his age. Family studies showed that his father carried a heterozygous TPO mutation p.T561M with no thyroid phenotype (figure 2). Unfortunately, his mother refused to provide a DNA sample. Since biallelic TPO mutations cause goitre and PCH, and the mother was euthyroid with a normal-sized thyroid gland, we speculate that patient 2 had inherited one of the heterozygous variants (p.R648Q/p.T561M) from his mother, and the other

Figure 3 Multiple sequence alignment of TPO from different species. The arrow indicates that the threonine 561 residue is located within a highly conserved region.

Figure 4 Structural analysis on p.Thr561Met. Residues around Thr561 at a distance of 10 Å were shown. (A) The wild type of Thr561. (B) The mutation type of Met561. The arrow indicates that the substitution, p.T561M, breaks two hydrogen bonds with an asparagine in position 557 and another hydrogen bond with a glutamic acid in position 558.
DISCUSSION

In the present study, we conducted one of the largest TPO gene mutation screenings, in a cohort of 192 patients with CH, in the Guangxi Zhuang Autonomous Region, China. The results of our study identified three different TPO variations in two patients with PCH with enlarged thyroid gland—which was in agreement with the previous notion that defects in TPO cause goitrous PCH—and revealed a 1% of TPO mutation rate among patients with CH. The mutation rate is similar to that of Korea\textsuperscript{18} and Iran,\textsuperscript{19} but lower than the rates reported for Portuguese patients with CH,\textsuperscript{20} and Turkish,\textsuperscript{21} Pakistani\textsuperscript{21} and Slovene\textsuperscript{22} patients with TDH. The difference may be explained by the target population and methods involved. Moreover, genetic diversity in each ethnic group may also be implicated.

The present study identified a novel variation: p.T561M in the TPO; and four known mutations: c.2422delT and p.R648Q in TPO, and p.R683L and p.L1343F in DUOX2. Cangül\textsuperscript{23} et al demonstrated that a homozygous deletion (c.2422delT) in the carboxy-terminal coding region of the TPO gene could result in a frameshift mutation and lead to an early stop codon in exon 14 of the gene (p.Cys808AlafsX24), thus causing CH. Pannain\textsuperscript{24} et al examined three patients with CH who were heterozygotes for two TPO gene mutations (p.E799K and p.R648Q). Satoh described an 8-year-old boy with normal newborn screening results who developed non-autoimmune hypothyroidism at the age of 1 year and 8 months, and it was found that the boy was heterozygous for R450H-TSHR mutation and double heterozygous for A1323T-DUOX2 and L1343F-DUOX2.\textsuperscript{25} In our recent study, we examined three patients with CH who had biallelic DUOX2 gene mutations (p.R683L and p.K530X) or triallelic DUOX2 gene mutations (p.R683L/p.R683L/p.L1343F and p.R683L/p.R683L/p.E879K/p.A1138D).\textsuperscript{11} Structural and in silico functional analysis of the novel candidate missense TPO mutation p.T561M confirmed the causative nature.

Threonine in position 561 of the TPO molecule is a highly-conserved amino acid residue. This residue strongly interacts with two other well-conserved residues, asparagine 557 and glutamic acid 558, via hydrogen bonds. Substitution of a neutral polar residue such as threonine by a nonpolar methionine at position 561, destabilises and changes the local structure of protein and is expected to affect amino acid interactions between closer residues of the protein.

Most cases of CH associated with alterations in the TPO gene are caused by either homozygous or compound heterozygous mutations. In the present study, a mutation homozygous for c.2422delT TPO combined with double heterozygous DUOX2 mutations (p.R683L/p.L1343F) were identified in patient 1; and triallelic

variant was de novo or the patient had inherited both of the variants in the same allele.

Table 1

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age* (year)</th>
<th>Weight* (kg)</th>
<th>Length* (cm)</th>
<th>TSH* (mIU/L)</th>
<th>FT4* (pmol/L)</th>
<th>L-T4 (μg/kg/day)</th>
<th>Thyroid morphology</th>
<th>TPO variations</th>
<th>Variations in other genes</th>
<th>Clinical phenotype</th>
<th>Development/age (year)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Male</td>
<td>NS</td>
<td>3.0 (10th–25th)</td>
<td>47 (&lt;3rd)</td>
<td>&gt;100</td>
<td>1.92</td>
<td>12/6</td>
<td>Goitre</td>
<td>c.2422delT/</td>
<td>p.R683L/p.L1343F (DUOX2)</td>
<td>PCH</td>
<td>normal/3.1</td>
</tr>
<tr>
<td>2</td>
<td>Male</td>
<td>4.5</td>
<td>13.5 (&lt;3rd)</td>
<td>98 (&gt;3rd)</td>
<td>&gt;100</td>
<td>0.30</td>
<td>15/5</td>
<td>Goitre</td>
<td>p.R648Q/p.T561M</td>
<td>no</td>
<td>physical and mental retardation/7.0</td>
<td>PCH</td>
</tr>
</tbody>
</table>

\textsuperscript{*}Age, Weight, Length, TSH, FT4 at diagnosis. NS, Newborn Screening; PCH, permanent congenital hypothyroidism; TSH, thyroid-stimulating hormone.
TPO pathogenic variants (p.R648Q/p.T561M/p.T561M) were identified in patient 2. It demonstrated that three or even more variations in one or more CH associated genes can be found in a single patient, which, in combination, affect the phenotype of the individual. The phenotype of the two patients was severely affected, which was suggested by the extremely low plasma thyroid hormone concentrations, highly elevated TSH levels and high dosage of L-T4. Therefore, the coexistence of multiple mutations may have intensified the severity of the hypothyroid condition.

The genotype–phenotype correlation according to the type of mutation could not be clearly determined yet, because only two patients were identified to be mutation-positive participants and both had multiple variations. Our study demonstrates that the mutational effects from other CH genes should be taken into account when defining the relationships between mutation genotypes and clinical phenotypes.

In conclusion, we conducted one of the largest TPO mutation screenings, in a cohort of 192 patients with CH, in the Guangxi Zhuang Autonomous Region, China. We identified the prevalence of a TPO pathogenic variant to be 1% among patients with CH. Three or even more variations in one or more CH associated gene can be found in a single patient, and the coexistence of multiple mutations may have affected the severity of the hypothyroid condition. A novel TPO variation was reported, which expanded the TPO mutation spectrum and indicated that the TPO mutation rate is very low in the Guangxi Zhuang Autonomous Region, China.

REFERENCES

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