

# BMJ Open Mutation screening of the *TPO* gene in a cohort of 192 Chinese patients with congenital hypothyroidism

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**To cite:** Fu C, Xie B, Zhang S, *et al.* Mutation screening of the *TPO* gene in a cohort of 192 Chinese patients with congenital hypothyroidism. *BMJ Open* 2016;**6**:e010719. doi:10.1136/bmjopen-2015-010719

► Prepublication history for this paper is available online. To view these files please visit the journal online (<http://dx.doi.org/10.1136/bmjopen-2015-010719>).

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Received 1 December 2015  
Revised 26 March 2016  
Accepted 5 April 2016



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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 leucocytes. 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 triallelic 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.R648Q in *TPO*, c.2048G>T/p.R683L and c.4027C>T/p.L1343F in *DUOX2*.

**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 may, in combination, affect the phenotype of the individual. A novel *TPO* variation c.1682C>T/p.T561M was found, thereby expanding the mutational spectrum of the gene.

## INTRODUCTION

Congenital hypothyroidism (CH) is a common endocrine disorder with prevalence

## 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.

ranging from 1:2000 to 1:4000 newborns.<sup>1</sup> Apart from iodine deficiency, which is still a major cause for endemic hypothyroidism in neonates,<sup>2</sup> 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,<sup>3</sup> which account for the majority of cases (80–85%)<sup>3–4</sup>; and (2) abnormalities in thyroid hormone synthesis (dysmorphogenesis), which account for the remaining 15–20% of cases,<sup>5</sup> and are associated with mutations in *DUOX2*, *TG*, *TPO*, *SLC5A5*, *SLC26A4* and *IYD* genes.<sup>6</sup>

Defects in the human *TPO* (NM\_000547) gene are reported to comprise some of the most common causes of thyroid dysmorphogenesis (TDH).<sup>7–10</sup> *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.<sup>11</sup> 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 73 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  $\geq$ 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  $\sim$ 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 leucocytes, using QIAamp DNA Blood Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. A CH capture panel an Illumina Truseq Custom Amplicon V.1.5 kit, included 10 known CH-associated genes (*TPO*, *TSHR*, *PAX8*, *NKX2.1*, *FOXE1*, *DUOX2*, *TG*, *SLC5A5*, *SLC26A4* and *IYD*) with complete coding regions and flanking intronic regions; the *DUOXA2* gene was not included. The prepared sample library was sequenced employing an Illumina MiSeq instrument using MiSeq Reagent Kit V.2, 500-cycles (Illumina Inc, San Diego, California, USA).

Illumina Amplicon Viewer V.1.3 and MiSeq Reporter V.2.3 software were used for data analysis, and the SnpEff<sup>12</sup> was used for variant annotation. Polyphen 2<sup>13</sup> and MutationTaster<sup>14</sup> were used to evaluate the pathogenicity of the novel mutations. DNAMAN software V.8 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

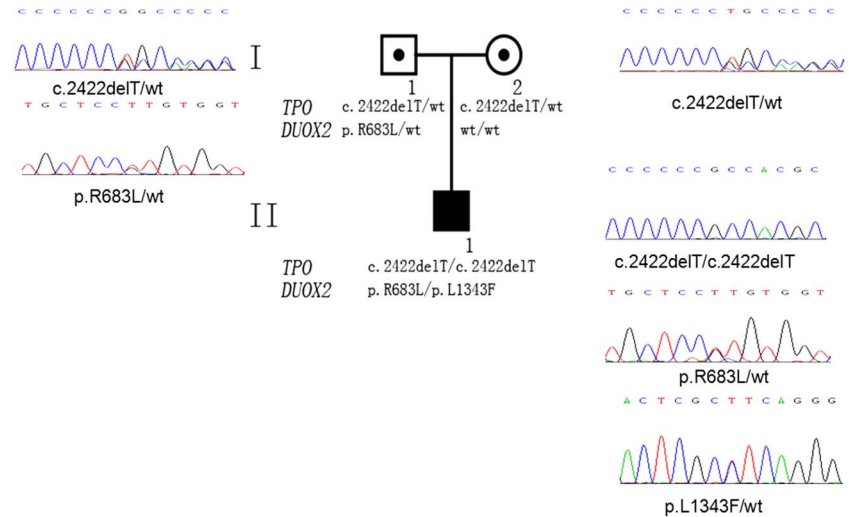
The crystal structure of human *TPO* is currently not available. To study the three-dimensional (3D) structure of the p.T561M *TPO*, we modelled the wild-type and mutant *TPO* variants in silico, using the Swiss Model program package.<sup>15 16</sup> Multiple templates were used to generate different target models, and the one presenting the best sequence identity percentage was chosen. Thus, a homology model was generated with human myeloperoxidase isoform 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 Viewer<sup>17</sup> 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  $>40\times$ . 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

**Figure 1** Pedigree of the affected family 1 and Sanger sequencing for validation of the mutations detected by the next-generation sequencing platforms.



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

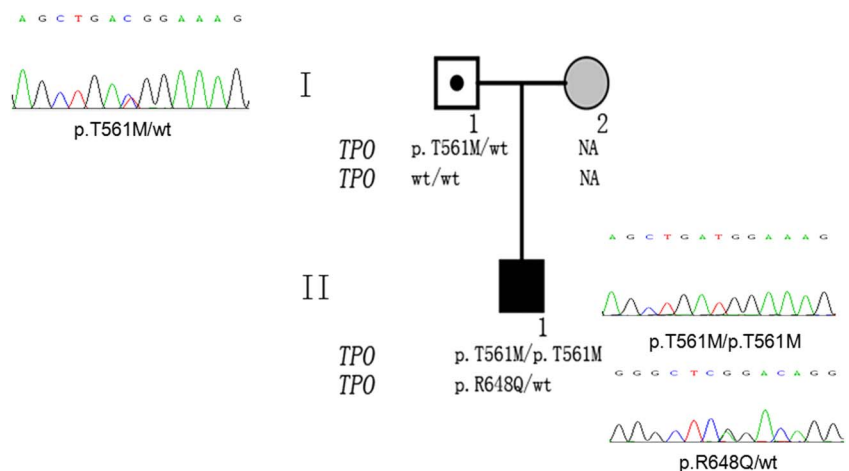
Lys627) residues located in the disallowed regions. However, these amino acid residues were distant from the active site. Consequently, the homology model was acceptable for subsequent analyses.

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.

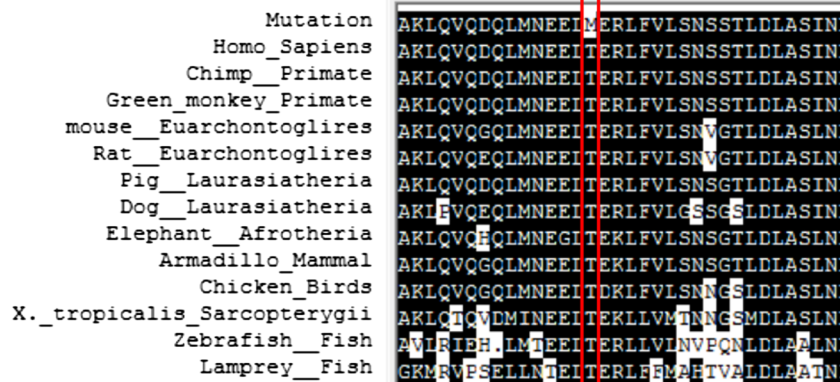
#### Clinical features and laboratory test results of the patients

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

**Figure 2** Pedigree of the affected family 2 and Sanger sequencing for validation of the mutations detected by the next-generation sequencing platforms. NA, data are not available.



**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.

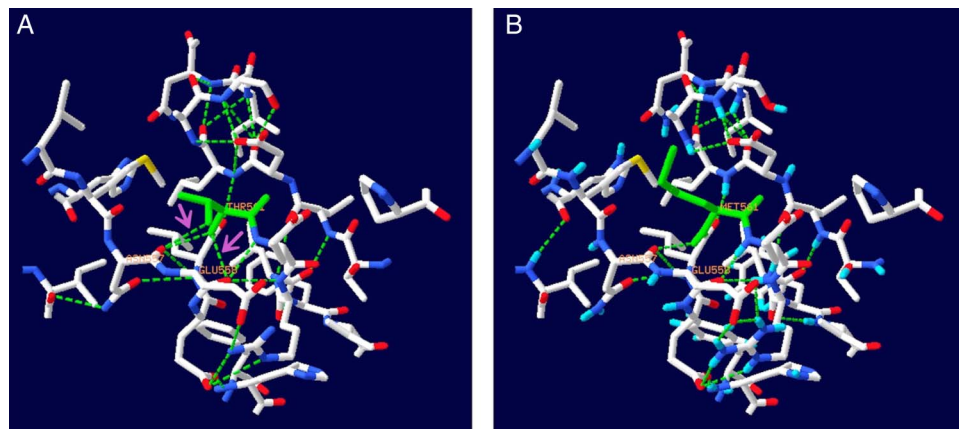


revealed a high level of TSH (78.6 mIU/L). He was therefore re-evaluated at hospital at the age of 13 days. A serum TSH level of >100 mIU/L (normal range 0.7–10 mIU/L), FT4 level of 1.92 pmol/L (normal range 12–32 pmol/L) and FT3 level of 1.68 pmol/L (normal range 2.65–9.68 pmol/L) confirmed the diagnosis. 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 (<3th) in height and 13.5 kg (<3th) 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 (<3th) and height 113 cm (<3th). 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 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.



**Table 1** Clinical features, laboratory results and TPO gene mutations in the two patients

Patient	Sex	Age* (year)	Weight* (kg) centile	Length* (cm) centile	TSH* (mIU/L)	FT4* (pmol/L)	L-T4(µg/kg/day)		Thyroid morphology	TPO variations	variations in other genes	Clinical phenotype	Development/age (year)
							initial	current					
1	Male	NS	3.0 (10th–25th)	47 (<3th)	>100	1.92	12/6		Goitre	c.2422delT/ c.2422delT	p.R683L/p. L1343F (DUOX2)	PCH	normal/3.1
2	Male	4.5	13.5 (<3th)	98 (<3th)	>100	0.30	15/5		Goitre	p.R648Q/p. T561M/p. T561M	no	PCH	physical and mental retardation/ 7.0
			Normal values	Normal values	0.27–10	12–22							

\*Age, Weight, Length, TSH, FT4 at diagnosis.

NS, Newborn Screening; PCH, permanent congenital hypothyroidism; TSH, thyroid-stimulating hormone.

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

## 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<sup>18</sup> and Iran,<sup>19</sup> but lower than the rates reported for Portuguese patients with CH,<sup>20</sup> and Turkish,<sup>21</sup> Pakistani<sup>21</sup> and Slovene<sup>22</sup> 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 *et al*<sup>23</sup> demonstrated that a homozygous deletion (c.2422delT) in the carboxyl-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 *et al*<sup>24</sup> 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*.<sup>25</sup> 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.E879K/p.A1138D).<sup>11</sup> 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

*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.

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**Contributors** CF and BX participated in the research design, performed the experiments, analysed the data and wrote the paper. SZ, JW, SL, RC, XF and JL collected clinical samples and analysed the clinical data. BX and HZ performed the three-dimensional analyses and interpreted the data. XG and SC proposed the research design, analysed the molecular and overall data, and participated in writing the paper.

**Funding** This study was supported by the National Natural Science Foundation of China (81260126), Key Projects of Guangxi Health Department (2012025) and Guangxi Natural Science Foundation Programme (2012GXNSFAA053174).

**Competing interests** None declared.

**Patient consent** Obtained.

**Data sharing statement** No additional data are available.

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