

# BMJ Open Prevalence of c.2268dup and detection of two novel alterations, c.670\_672del and c.1186C>T, in the *TPO* gene in a cohort of Malaysian–Chinese with thyroid dysmorphonogenesis

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## ABSTRACT

**Objectives:** The c.2268dup mutation in the thyroid peroxidase (*TPO*) gene is the most common *TPO* alteration reported in Taiwanese patients with thyroid dysmorphonogenesis. The ancestors of these patients are believed to originate from the southern province of China. Our previous study showed that this mutation leads to reduced abundance of the *TPO* protein and loss of *TPO* enzyme activity in a Malaysian–Chinese family with goitrous hypothyroidism. The aim of our study was to provide further data on the incidence of the c.2268dup mutation in a cohort of Malaysian–Chinese and its possible phenotypic effects.

**Setting:** Cohort study.

**Participants:** Twelve biologically unrelated Malaysian–Chinese patients with congenital hypothyroidism were recruited in this study. All patients showed high thyrotropin and low free thyroxine levels at the time of diagnosis with proven presence of a thyroid gland.

**Primary outcome measure:** Screening of the c.2268dup mutation in the *TPO* gene in all patients was carried out using a PCR–direct DNA sequencing method.

**Secondary outcome measure:** Further screening for mutations in other exonic regions of the *TPO* gene was carried out if the patient was a carrier of the c.2268dup mutation.

**Results:** The c.2268dup mutation was detected in 4 of the 12 patients. Apart from the c.2268dup and a previously documented mutation (c.2647C>T), two novel *TPO* alterations, c.670\_672del and c.1186C>T, were also detected in our patients. In silico analyses predicted that the novel alterations affect the structure/function of the *TPO* protein.

**Conclusions:** The c.2268dup mutation was detected in approximately one-third of the Malaysian–Chinese patients with thyroid dysmorphonogenesis. The detection of the novel c.670\_672del and c.1186C>T alterations expand the mutation spectrum of *TPO* associated with thyroid dysmorphonogenesis.

## INTRODUCTION

Congenital hypothyroidism (CH) is one of the most common endocrine disorders in

## Strengths and limitations of this study

- The c.2268dup mutation was detected in approximately one-third of the Malaysian–Chinese with thyroid dysmorphonogenesis. c.2268dup in the homozygous form might be associated with the phenotype of dysmorphonogenetic goitre.
- Two novel thyroid peroxidase (*TPO*) alterations, c.670\_672del and c.1186C>T, were also detected in this study. In silico analyses revealed that the two alterations may affect the normal structure/function of the mutant *TPO* protein.
- The in silico functional analyses could not be further validated because of unavailability of thyroid tissue samples from the patients.

the world affecting 1 in 3000–4000 newborn babies, with 10–20% of the cases being due to thyroid dysmorphonogenesis.<sup>1</sup> Over the past three decades, numerous cases of dysmorphonogenetic CH have been linked to alterations in the thyroid peroxidase (*TPO*) gene.<sup>2–3</sup> This gene encodes a protein 933 amino acids in length, which plays an important role in thyroid hormone synthesis.<sup>4</sup> Niu *et al*<sup>5</sup> in 2002 reported a nonsense mutation, c.2268dup, a common cause of dysmorphonogenetic CH in Taiwan with molecular proof of a founder effect. Recently, we identified the c.2268dup mutation in a Malaysian–Chinese family with goitrous CH and showed that the mutation leads to the reduction in *TPO* protein expression with a consequential loss of enzyme activity.<sup>6</sup> Chinese form the second largest ethnic group, which constitutes about 24.6% of the 28.3 million Malaysian population.<sup>7</sup> As the cause of dysmorphonogenetic CH in Malaysian–Chinese remains unclear, we embarked on this study with the aim of providing further data on the

incidence of the c.2268dup mutation in Malaysian-Chinese and its possible phenotypic effects.

## SUBJECTS AND METHODS

### Subjects

A cohort (duration of follow-up 3–25 years) of 12 biologically unrelated Chinese patients with dysmorphogenetic CH who attended the Paediatric Endocrine Clinic, University Malaya Medical Centre (UMMC) was recruited for this study. None of the patients were from a consanguineous family. All patients had high thyrotropin and low free thyroxine levels at the time of diagnosis, with proven presence of a thyroid gland (table 1). Serum thyroglobulin level was measured in patients who had reached puberty (12 years and older) or presented with goitre, except for patients CHP51 and CHP55 who were transferred to adult care and another hospital, respectively (table 1). Informed written consent was obtained either from the patient or their parent/guardian. This study was approved by the UMMC Ethics Committee (Institutional Review Board) in accordance with the ICH-GCP guideline and the Declaration of Helsinki (reference number 654.16). The perchlorate discharge test was, however, not performed in our patients, since permission was not granted by the majority of the patients' parents.

### TPO mutation screening

PCR amplification and direct DNA sequencing were performed to screen for alterations in the *TPO* gene using genomic DNA extracted from peripheral venous blood. A forward (5'-ACAGGGACGTTGGTGTGTGG-3') and reverse (5'-TCAGAAGCACCTTTTGGCG-3') primer were used to PCR-amplify exon 13 of the *TPO* gene (NM\_000547.5) where the c.2268dup mutation is located. Further screening for mutations in other exonic

regions of the *TPO* gene<sup>8</sup> was carried out if the patient was a carrier of the c.2268dup mutation. To confirm that an alteration in the *TPO* gene is due to a disease-causing mutation rather than a polymorphism, a total of 100 chromosomes from 50 unrelated healthy individuals were also screened for the same mutation.

### In silico analysis of the novel c.670\_672del (p.Asp224del) and c.1186C>T (p.Arg396Cys) mutations

The effects of the novel mutations on normal TPO activity were evaluated using SIFT<sup>8a</sup> and Polyphen-2<sup>8b</sup> algorithms. Alignment of the human TPO sequence with those of mouse, rat, pig, dog and chicken was performed using CLC Sequence Viewer 6.5.2 software (CLC bio, Aarhus, Denmark). Homology models of human TPO including the wild-type and two mutant proteins (p.Asp224del and p.Arg396Cys) were generated, verified and compared as described previously.<sup>8</sup>

## RESULTS AND DISCUSSION

In addition to a patient with the homozygous c.2268dup mutation reported in our previous study,<sup>6</sup> c.2268dup was detected in 31% of all alleles studied. So far, the mutation has only been detected in patients with confirmed a total iodide organification defect (TIOD) tested using the perchlorate discharge test.<sup>5–9</sup> The test for TIOD was, however, not performed in our patients. This could be the reason for the higher prevalence of the c.2268dup mutation in the Taiwanese patients compared with our study.<sup>5–9</sup> Nonetheless, the difference in origin between the Chinese population of Malaysia<sup>10</sup> and Taiwan<sup>11</sup> may also have contributed to this variation. Further studies on the c.2268dup mutation by increasing the sample size and collecting information on the ancestral origins of the patients are expected to result in a deeper

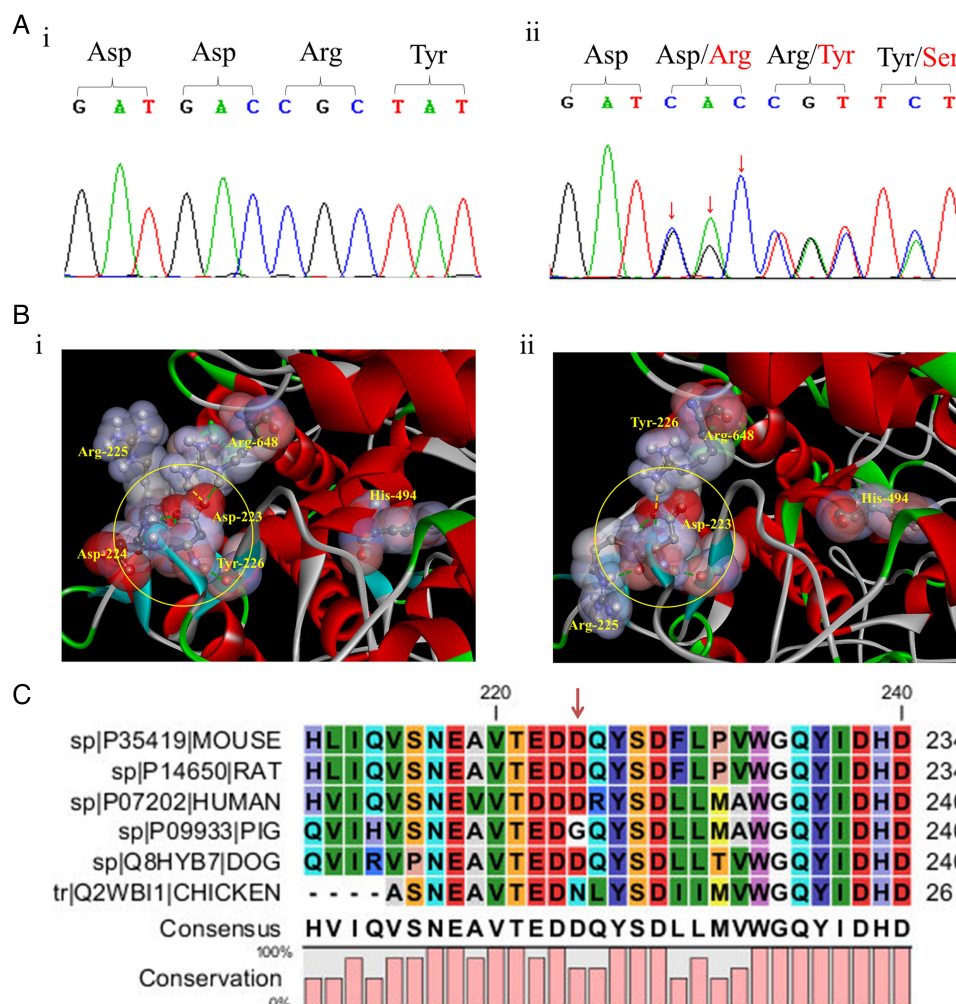
**Table 1** Profiles of Malaysian-Chinese patients with CH showing the respective thyroid function at the time of diagnosis

CH patient (CHP)	Gender	Age at diagnosis	Duration of follow-up (years)	TSH (μIU/mL)	fT <sub>4</sub> (pmol/L)	hTG (ng/mL)	Thyroid scan (at 3 years old)/ultrasonography
16	Female	10 days	17	26.5	11.3	40.7	Normal
18*	Male	3 weeks	20	59.0	13.0	26	Multinodular goitre at 13.5 years
21	Female	3 months	13	20.6	14.1	20	Normal
24	Female	Newborn	6	61.3	13.6	N/A	Normal
38*	Male	5 days	7	42.6	14.3	N/A	Normal
40	Male	4 days	10	27.8	18.4	N/A	Normal
45	Male	Newborn	6	217.0	5.0	N/A	Normal
51	Male	4 weeks	18	33.3	12.2	N/A	Normal
55	Male	7 weeks	11	>100	5.0	N/A	Normal
56	Female	5 weeks	25	181	5.0	2.6	Normal
58*	Male	5 days	7	37	19.9	N/A	Normal
59*	Male	3 months	15	>100	0.5	283	Normal

Reference ranges: TSH, cord=<25.0 μIU/mL; 1–3 days=2.5–13.0 μIU/mL; <1 month=0.6–10.0 μIU/mL; ≥1 month =0.6–8.0 μIU/mL. fT<sub>4</sub>, cord=28.4–68.4 pmol/L; 1 month=20.0–28.4 pmol/L; ≥4 months =9.0–24.5 pmol/L. hTG, 0–55.0 ng/mL. N/A, hTG level was not measured in patients younger than 12 years of age unless goitre was present. CHP51 and CHP55 were transferred to adult care and another hospital, respectively.

\*Patients with the c.2268dup mutation.

CH, congenital hypothyroidism; fT<sub>4</sub>, free thyroxine; hTG, human thyroglobulin; TSH, thyrotropin.



**Figure 1** (A) DNA sequencing profiles. Electropherogram profiles of a control with a wild-type allele (i) and CHP51 who is a heterozygote for the c.670\_672del mutation (ii). The three deleted nucleotides (GAC) are indicated by the arrows. The sequence alteration is predicted to produce an in-frame deletion of a single amino acid, aspartic acid (p.Asp224del). (B) Homology models illustrating the three-dimensional orientation of the wild-type (i) and mutant p.Asp224del (ii) thyroid peroxidase (TPO) proteins. The protein backbones are presented as ribbons ( $\alpha$ -helix in red,  $\beta$ -pleated sheet in cyan, coils in grey, and turns in green). Hydrogen bonds are highlighted in: (1) green, hydrogen bond under the normal criteria; (2) brown, hydrogen bond/salt bridge which forms between the O atom of the carboxylate group and the H atom of an ammonium group in highly charged regions. Residues Arg-223 to Tyr-226, Arg-648 and His-494 (iron-binding site) are represented as a Connolly surface to allow the visualisation of the conformational changes in the TPO protein and its binding pocket. The Connolly surface is coloured according to electrostatic potential spectrum (negative potential in red, to neutral in white, to positive in blue). Regions in yellow rings highlight the interrupted hydrogen bond network observed when the wild-type (i) changes to the mutant (ii) TPO protein. (C) Multiple-sequence alignment of human TPO with TPO of mouse, rat, pig, dog and chicken. The alignment data show that the negatively charged region (Asp-222, Asp-223 and Asp-224) is conserved among human and many different animal species. The position of the deleted residue (p.Asp224del) is indicated by the arrow.

understanding of the frequency and distribution pattern of the c.2268dup mutation in the Malaysian–Chinese population. Two patients in this study, CHP18 and CHP59, were homozygotes, while another two, CHP38 and CHP58, were heterozygotes for the mutation.

Apart from the c.2268dup mutation, a novel, heterozygous c.670\_672del mutation in exon 7 of the *TPO* gene was detected in patient CHP58. The deletion of three nucleotides (GAC) is predicted to produce an in-frame deletion of a single aspartic acid (p.Asp224del) in the TPO protein (figure 1A). Three-dimensional (3-D) model analysis of the mutant protein revealed that the

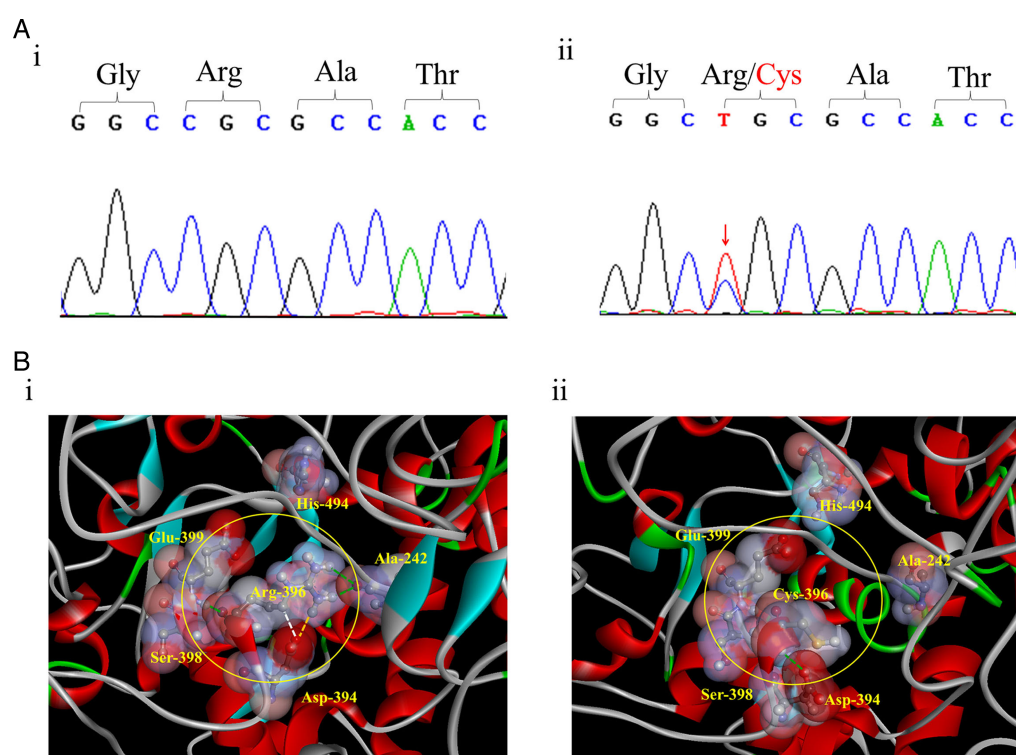
deleted Asp-224 residue is located within a  $\beta$ -strand. The mutation has caused conformational changes in the protein by shortening the length of the  $\beta$ -strand and also disrupted the correct orientation of the hydrogen bond network between Asp-223 with Asp-224, Arg-225, Tyr-226 and Arg-648. Since the altered sites are located so close to His-494, a proposed iron (haem axial ligand)-binding site,<sup>12</sup> it could possibly interfere with binding of the iron ion at His-494 or the electron transfer activity of TPO in which His-494 is the source of the electron.<sup>13</sup> It is also predicted that the deletion of Asp-224 will alter the orientation of a salt bridge between Asp-223 and Arg-648



(figure 1B). Interestingly, three aspartic acid residues, Asp-222, Asp-223 and Asp-224, present in the same  $\beta$ -strand that is located on the outer surface of the wild-type TPO, contribute to a highly negatively charged region, which is also conserved across many species including mice, rat and dog, implying that this region is crucial for the normal activity of the protein (figure 1C).

Most cases of CH associated with alterations in the *TPO* gene are caused by either homozygous or compound heterozygous mutations. In the present study, three different alterations in the *TPO* gene were identified in CHP38 other than the confirmed polymorphism. In addition to the c.2268dup, a novel, heterozygous mutation, c.1186C>T (p.Arg396Cys), was detected in exon 8 and is expected to cause substitution of cysteine for arginine at codon 396 (figure 2A). Results from both SIFT and Polyphen-2 analyses indicated that the substitution is damaging, implying that this residue is important in the structure/function of TPO. A study has shown that Arg-396 is one of the important amino acids which could be involved in stabilising the transition state of TPO protein during catalytic intermediate formation.<sup>14</sup> The formation of a stable catalytic

intermediate (compound I) of TPO with  $H_2O_2$  is crucial for thyroid hormone synthesis.<sup>15 16</sup> The catalytic process is initiated by diffusion of  $H_2O_2$  into the active site of the TPO protein. The  $\alpha$ -nucleophile,  $H_2O_2$ , donates a proton to the distal imidazole ring (His-239) to form a bond with the iron ion bound to residue His-494. After binding has taken place, the protein attains the transition state to form compound I. The arginine at position 396 is believed to play a role in stabilising the charge for the transition state of the protein through electrostatic interaction.<sup>14 17</sup> Alternatively, it is believed that the arginine contributes to the abnormally low pKa value of the distal histidine in the native resting enzyme. The changes in pKa value in the transition state of the distal imidazole are the key to the effectiveness of the catalytic process/rate of compound I formation.<sup>17</sup> Therefore, substitution of cysteine for arginine can bring devastating effects to protein stability. In the present study, the 3-D model analysis showed that the p.Arg396Cys mutation led to structure alteration through modification of the hydrogen bond network in the hydrophobic pocket, which might interfere with haem binding at Glu-399<sup>18</sup> (figure 2B).



**Figure 2** (A) DNA sequencing profiles. Electropherogram profiles of a control with a wild-type allele (i) and CHP38 who is a heterozygote for the c.1186C>T mutation (ii). The single-nucleotide transition is indicated by the arrow. The sequence alteration is predicted to cause the substitution of cysteine for arginine at codon 396 (p.Arg396Cys). (B) Homology models illustrating the three-dimensional orientation of the wild-type (i) and mutant p.Arg396Cys (ii) thyroid peroxidase (TPO) proteins. The protein backbones are presented as ribbons ( $\alpha$ -helix in red,  $\beta$ -pleated sheet in cyan, coils in grey, and turns in green). Hydrogen bonds are highlighted in: (1) green, hydrogen bond under the normal criteria; (2) brown, hydrogen bond/salt bridge which forms between the O atom of the carboxylate group and the H atom of an ammonium group in highly charged regions; (3) white, hydrogen bond between O atom of the carboxylate group and H atom on an electro-positive C atom. Residues Ala-242, Arg-396/Cys-396, Ser-398, Glu-399 (haem-binding site) and His-494 (iron-binding site) are represented as a Connolly surface to allow the visualisation of the conformational changes in the TPO protein and its binding pocket. The Connolly surface is coloured according to the electrostatic potential spectrum (negative potential in red, to neutral in white, to positive in blue). Regions in yellow rings highlight the interrupted hydrogen bond network observed when the wild-type (i) changes to the mutant (ii) TPO protein.



Apart from the c.2268dup and c.1186C>T mutations, a non-synonymous substitution, c.2647C>T, was also identified in exon 16 of CHP38. The nucleotide alteration leads to substitution of serine for proline at codon 883 in the C-terminal tail (Val-869 to Leu-933) of the TPO protein and has previously been reported in patients with dyshormonogenetic CH in populations of Korea<sup>19</sup> and Japan.<sup>20</sup> However, the consequence of the c.2647C>T mutation remains ambiguous, since both SIFT and Polyphen-2 analyses<sup>3</sup> indicate that this alteration is 'benign', suggesting that Pro-883 is probably not critical for structure/function of the TPO and could be a rare polymorphism rather than a disease-causing allele.

It is worth noting that CHP18 and the two sisters with homozygous c.2268dup reported in our previous study<sup>6</sup> developed large multinodular goitres in their mid or late adolescent years. Although it is not known whether the reduction in TPO expression due to c.2268dup can lead to increased risk of malignant transformation, other studies have shown that cases of thyroid carcinoma have developed from congenital goitres that are associated with TPO mutation<sup>21, 22</sup> or with lower/absence of TPO expression.<sup>23, 24</sup> Therefore, careful surveillance for potential thyroid neoplasm in patients with c.2268dup mutation is important.

## CONCLUSION

In conclusion, we report two novel alterations in the TPO gene, c.670\_672del and c.1186C>T, which are probably pathogenic, and an association of c.2268dup mutation with approximately one-third of a cohort of Malaysian-Chinese with dyshormonogenetic CH. This study also supports our previous findings that c.2268dup homozygotes developed dyshormonogenetic goitre in their mid or late adolescent years. These data will be useful in diagnosing or predicting goitrous dyshormonogenetic CH.

**Contributors** CCL participated in the research design, performed the experiments, analysed the data and wrote the paper; FH and MYJ collected clinical samples and analysed the clinical data; CHH and RO performed the 3-D analyses and interpreted the data; SMJ proposed the research design, analysed the molecular and overall data, and participated in writing the paper.

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**Competing interests** None.

**Patient consent** Obtained.

**Ethics approval** The University of Malaya Medical Centre (UMMC) Ethics Committee.

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**Data sharing statement** Extra data can be accessed via the Dryad data repository at <http://datadryad.org/with/doi:10.5061/dryad.5230v>.

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