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Research**Identification of two novel mutations, c.670_672del and c.1186C>T, and the prevalence of c.2268dup in the *TPO* gene in a cohort of Malaysian-Chinese with thyroid dyshormonogenesis**

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ABSTRACT

Objectives: The c.2268dup mutation in the thyroid peroxidase (*TPO*) gene is the most common *TPO* defect reported in Taiwanese patients with thyroid dysmorphogenesis. 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 *TPO* gene mutation in a cohort of Malaysian-Chinese and its possible phenotypic effects.

Setting: Cohort study.

Participants: Twelve unrelated Malaysian-Chinese patients with congenital hypothyroidism were recruited in this study. All patients showed high TSH and low fT_4 levels at the time of diagnosis with proven presence of thyroid gland

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

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

Results: The c.2268dup mutation was detected in 4 out of the 12 patients. A homozygous patient, CHP59 had developed large multinodular goiter at the age of 13.5 years which supports our previous findings that c.2268dup homozygotes developed dysmorphogenetic goiter in their mid or late adolescent years. Apart from the c.2268dup and a documented mutation, c.2647C>T, two novel *TPO* mutations namely c.670_672del and c.1186C>T were also detected in our patients. *In silico* analyses predicted that the novel mutations affect the structure/function of the *TPO* protein.

Conclusion: The c.2268dup was detected in approximately one third of the Malaysian-Chinese with thyroid dysmorphogenesis. The detection of the novel c.670_672del and

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3 51 c.1186C>T alterations expand the mutation spectrum of *TPO* associated with thyroid
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5 52 dysmorphogenesis.
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10 54 **Strengths and limitations of this study**

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13 55 • The c.2268dup was detected in approximately one third of the Malaysian-Chinese
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15 56 with thyroid dysmorphogenesis. The c.2268dup in the homozygous form might be
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17 57 associated with the phenotype of dysmorphogenetic goiter.
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19 58 • Two novel *TPO* mutations namely c.670_672del and c.1186C>T were also detected
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21 59 in this study. *In silico* analyses revealed that the two mutations may affect the normal
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23 60 structure/function of the mutant TPO protein.
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25 61 • The *in silico* functional analyses could not be further validated due to unavailability of
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27 62 thyroid tissue samples from the patients.
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68 **Keywords:** Congenital hypothyroidism, *thyroid peroxidase*, c.2268dup, novel mutations,
69 Malaysian-Chinese
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71 INTRODUCTION

72 Congenital hypothyroidism (CH) is one of the most common endocrine disorders in
73 the world affecting 1 in 3000 - 4000 newborn babies where 10 - 20 % of the cases were due
74 to thyroid dysmorphogenesis [1]. Over the past three decades, numerous cases of
75 dysmorphogenetic CH have been linked to defects in the *TPO* gene [2]. This gene encodes a
76 protein with 933 amino acids in length which plays an important role in thyroid hormones
77 synthesis [3]. Niu *et al.* in 2002 reported a nonsense mutation, c.2268dup, a common cause of
78 dysmorphogenetic CH in Taiwan with molecular proof of a founder effect where the
79 ancestors of these patients originated from the southern province of China [4]. Recently, we
80 identified the c.2268dup mutation in a Malaysian-Chinese family with goitrous CH and
81 showed that the mutation leads to the reduction of TPO protein expression with a
82 consequential loss of enzyme activity [5]. The Chinese forms the second largest ethnic group
83 which constitutes about 24.6 % of the 28.3 million Malaysian populations [6]. As the cause of
84 dysmorphogenetic CH in Malaysian-Chinese remains unclear, we therefore embarked on
85 this present study with the aim of providing further data on the incidence of *TPO* gene
86 mutation in Malaysian Chinese and its possible phenotypic effects.

88 SUBJECTS AND METHODS

89 *Subjects for the TPO mutation screening*

90 A cohort (duration of follow-up between 3 and 25 years) of 12 unrelated Chinese
91 patients with dysmorphogenetic CH who attended the Paediatric Endocrine Clinic,
92 University Malaya Medical Centre (UMMC) was recruited for this study. Mutational
93 screening was performed on genomic DNA extracted from peripheral venous blood of all
94 patients who had high TSH and low fT₄ levels at the time of diagnosis with proven presence
95 of thyroid gland (Table S1). A forward (5'-ACAGGGACGTTGGTGTGTGG-3') and a

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3 96 reverse (5'-TCAGAAGCACCTTTTGGCG-3') primer were used to PCR-amplify exon 13 of
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5 97 the *TPO* gene (NM_000547.5) where the c.2268dup mutation is located. Further screening
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7 98 for mutations in other exonic regions of the *TPO* gene [7] was carried out if the patient is a
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9 99 carrier for the c.2268dup mutation. To confirm that an alteration in the *TPO* gene is due to a
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11 100 disease-causing mutation instead of a polymorphism, a total of 100 chromosomes from 50
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13 101 unrelated healthy individuals were also screened for the same mutation. Informed written
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15 102 consent was obtained either from patients or their parent/guardian. This study was approved
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17 103 by the UMMC Ethical Committee (Institutional Review Board) in accordance to the ICH-
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19 104 GCP guideline and the Declaration of Helsinki (Reference number, 654.16).

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21 105 *In silico analyses of the novel c.670_672del (p.Asp224del) and c.1186C>T (p.Arg396Cys)*
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23 106 *mutations*

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29 107 The effects of the novel mutations on normal TPO activity were evaluated using SIFT
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31 108 and Polyphen-2 algorithms. Alignment of human TPO sequence with those of mouse, rat, pig,
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33 109 dog and chicken was performed using CLC Sequence Viewer 6.5.2 software (CLC bio,
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35 110 Aarhus, Denmark). The homology models of human TPO including the wild type and the two
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37 111 mutant proteins: p.Asp224del and p.Arg396Cys were generated, verified and compared as
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39 112 described before [7].
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46 114 **RESULTS AND DISCUSSION**

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49 115 The c.2268dup mutation was detected in 4 out of the total 12 unrelated patients in this
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51 116 study. Two patients, CHP18 and CHP59, were homozygotes while another two, CHP38 and
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53 117 CHP58, were heterozygotes for the mutation. In addition to a case reported in our previous
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55 118 study [5], the c.2268dup was detected in only 31 % of the total alleles studied, lower than
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3 119 what were reported in the studies by Niu *et al.* (2002) (86 %) [4] and Wu *et al.* (2002) (40 %)
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5 120 [8]. For their study, only patients with confirmed total iodide organification defect (TIOD)
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7 121 through perchlorate discharge test were screened for the *TPO* gene mutations. In contrast, the
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9 122 perchlorate test was not performed in our patients since permission was not granted by the
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11 123 majority of the patients' parents. This could be the reason for the higher prevalence of the
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13 124 c.2268dup mutation in the Taiwanese patients when compared to our study. Nonetheless, the
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15 125 difference in the origin between the Chinese population of Malaysia [9] and Taiwan [10] may
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17 126 also have contributed to this variation.
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23 128 Apart from the c.2268dup mutation, a novel c.670_672del mutation in exon 7 of the
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25 129 *TPO* gene was detected in patient CHP58. The deletion of 3 nucleotides (GAC) is predicted
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27 130 to produce an in-frame deletion of a single aspartic acid (p.Asp224del) in the TPO protein
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29 131 (Fig. 1A). Three dimensional (3-D) model analysis of the mutant protein revealed that the
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31 132 deleted Asp-224 residue is located within a beta-strand. The mutation has brought
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33 133 conformational changes to the protein by shortening the length of the beta-strand and also
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35 134 disrupted the correct orientation of hydrogen bonds network between Asp-223 with Asp-224,
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37 135 Arg-225, Tyr-226 and Arg-648. Since the altered sites are located so close to His-494, a
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39 136 proposed iron (heme axial ligand) binding site [11], it could possibly interfere with binding of
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41 137 the iron ion at His-494 or the electron transfer activity of TPO where His-494 is the source of
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43 138 the electron [12]. It is also predicted that the deletion of Asp-224 will alter the orientation of a
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45 139 salt bridge between Asp-223 and Arg-648 (Fig. 1B). Interestingly, three aspartic acid residues:
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47 140 Asp-222, Asp-223 and Asp224 present in the same beta-strand that is located on the outer
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49 141 surface of the wild type TPO contribute to a highly negatively-charged region which is also
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51 142 conserved across many species including mice, rat and dog implying that this region is
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53 143 crucial for the normal activity of the protein (Fig. 1C).
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3 144 Most cases of CH associated with defects in the *TPO* gene were caused by either
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5 145 homozygous or compound heterozygous mutations. In the present study, three different
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7 146 mutations in *TPO* gene were identified in CHP38. In addition to the c.2268dup, a novel
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9 147 mutation, c.1186C>T (p.Arg396Cys), was detected in exon 8 and is expected to cause a
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11 148 substitution of arginine to cysteine at codon 396 (Fig 2A). Results from both SIFT and
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14 149 Polyphen-2 analyses indicated that the substitution is damaging and hence implying that this
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16 150 residue is important in the structure/function of the TPO. A study has shown that the Arg-396
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18 151 is one of the important amino acids which could be involved in stabilizing the transition state
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20 152 of TPO protein during the catalytic intermediate formation [13]. The formation of a stable
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22 153 catalytic intermediate (compound I) of the TPO with H₂O₂ is crucial for thyroid hormone
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24 154 synthesis [14-15]. Catalytic process is initiated by the diffusion of H₂O₂ into the active site of
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26 155 the TPO protein. The α -nucleophile H₂O₂ donates a proton to the distal imidazole ring (His-
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28 156 239) to form a bond with the iron ion bound to residue His-494. After binding takes place, the
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30 157 protein attains transition state to form compound I. The arginine at position 396 is believed to
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32 158 play a role in stabilizing the charge for transition state of the protein through electrostatic
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34 159 interaction [13,16]. Alternatively, it is believed that the arginine contributes to the abnormally
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36 160 low pKa value of the distal histidine in the native resting enzyme. The changes of the pKa
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38 161 value in the transition state of the distal imidazole are the key to determine the effectiveness
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40 162 of the catalysis process/rate of the compound I formation [16]. Therefore, a substitution from
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42 163 arginine to cysteine can bring devastating effects to the protein stability. In the present study,
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44 164 the 3-D model analysis showed that the p.Arg396Cys mutation has led to the structure
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46 165 alteration through the modification of the hydrogen bond network in the hydrophobic pocket
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48 166 which might interfere with the heme binding at Glu-399 [17] (Fig. 2B).

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52 167 Apart from the c.2268dup and c.1186C>T mutations, a c.2647C>T mutation was also
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54 168 identified in exon 16 of CHP38, where it leads to a substitution of proline to serine at codon
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3 169 883 in the C-terminal tail (Val-869 to Leu-933) of the TPO protein. SIFT and Polyphen-2
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5 170 analyses result from a previous study [2] indicates that this mutation is “benign” suggesting
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7 171 that the Pro-883 is probably not critical for structure/function of the TPO and does not
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10 172 modify TPO function. Interestingly, this mutation had also been reported in populations of
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12 173 Korea [18] and Japan [19]. Whether the c.2647C>T mutation is possibly a rare polymorphism
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14 174 is not known. Despite the *in silico* findings indicated that the substitution of Pro-883 to serine
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16 175 might not be critical to the protein structure/function, previously reported cases showed that
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18 176 patients associated with this mutation presented with severe dysmorphogenetic CH [18-19].
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20 177 Since Human Splicing Finder (HSF) analysis predicted that the c.2647C>T mutation
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22 178 interrupts the sequences recognized by ESE proteins (data not shown), we therefore could not
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24 179 exclude that the sequence alteration might possibly regulate the splicing activity of the TPO
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26 180 pre-mRNA and lead to CH in these patients.
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34 182 It is worth noting that patients with homozygous c.2268dup inclusive of two patients
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36 183 reported in our previous study [5], except for patient CHP59 who was 12-year old at the time
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38 184 of the study, had developed large multinodular goiter in their mid or late adolescent years.
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40 185 Although it is not known whether the reduction in TPO expression due to c.2268dup can lead
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42 186 to increased risk of malignant transformation, other studies have shown cases of thyroid
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44 187 carcinoma has developed from congenital goiter that is associated with *TPO* mutation [20-21]
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46 188 or lower/ absence of TPO expression [22-23]. Therefore, it is important to have a careful
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48 189 surveillance for potential thyroid neoplasm in patients with c.2268dup mutation.
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52 191 **CONCLUSION**

53
54 192 In conclusion, we report two novel mutations in the *TPO* gene, c.670_672del and
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56 193 c.1186C>T, and reveal the association of c.2268dup mutation with approximately one third
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3 194 of a cohort of Malaysian-Chinese with dysmorphogenetic CH. This study also support our
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5 195 previous findings that c.2268dup homozygotes developed dysmorphogenetic goiter in their
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7 196 mid or late adolescent years. These data will be useful in diagnosing or predicting goitrous
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10 197 dysmorphogenetic CH.
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3 199 **Contributors:**

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5 200 CCL participated in research design, performed the experiments, analyzed the data and wrote
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7 201 the paper; FH and MYJ collected clinical samples and analyzed the clinical data; CHH and
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9 202 RO performed the 3D analyses and interpreted the data; SMJ proposed research design,
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11 203 analyzed the molecular and overall data, and participated in writing the paper.
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21
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27 210 **Competing interests:** None

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31 212 **Ethics approval:** The University of Malaya Medical Centre (UMMC) Ethical Committee
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36 214 **Data sharing statement:** No additional data are available
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291 **FIGURE-LEGEND**

292 **Figure 1:** (A) DNA sequencing profiles. Electropherogram profiles of a control with a wild
293 type allele (i), and CHP51 who is a heterozygote for the c.670_672del mutation (ii). The three
294 deleted nucleotides (GAC) are indicated by the arrows. The sequence alteration is predicted
295 to produce an in-frame deletion of a single amino acid, aspartic acid (p.Asp224del). (B)
296 Homology models illustrating the 3-D orientation of the wild type (i) and mutant
297 p.Asp224del (ii) TPO proteins. The protein backbones are presented as ribbons (alpha-helix
298 in red, beta-pleated sheet in cyan, coils in grey, and turns in green). Hydrogen bonds are
299 highlighted in: 1) green, hydrogen bond under the normal criteria, 2) brown, hydrogen
300 bond/salt bridge which forms between the O atom of the carboxylate group and the H atom of
301 an ammonium group in highly charged region. Residues Arg-223 to Tyr-226, Arg-648 and
302 His-494 (iron binding site) are represented as Connolly surface to allow the visualization of
303 the conformational changes in the TPO protein and its binding pocket. The Connolly surface
304 is colored according to electrostatic potential spectrum (negative potential, in red, to neutral,
305 in white, to positive, in blue). Regions in yellow rings highlight the interrupted hydrogen
306 bond network observed when the wild type (i) changes to the mutant TPO protein (ii).
307 (C) Multiple-sequence alignment of human TPO with those of mouse, rat, pig, dog and
308 chicken. The alignment data show that the negatively charge region (Asp-222, Asp-223 and
309 Asp224) is conserved amongst human and many different animal species. The position of the
310 deleted residue (p.Asp224del) is indicated by the arrow.

311 **Figure 2**

312 (A) DNA sequencing profiles. Electropherogram profiles of a control with a wild type allele
313 (i), and CHP38 who is a heterozygote for the c.1186C>T mutation (ii). The single nucleotide
314 transition is indicated by the arrow. The sequence alteration is predicted to cause a
315 substitution of arginine to cysteine at codon 396 (p.Arg396Cys). (B) Homology models

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3 316 illustrating the 3-D orientation of the wild type (i) and mutant p.Arg396Cys (ii) TPO proteins.
4
5 317 The protein backbones are presented as ribbons (alpha-helix in red, beta-pleated sheet in cyan,
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7 318 coils in grey, and turns in green). Hydrogen bonds are highlighted in: 1) green, hydrogen
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9 319 bond under the normal criteria, 2) brown, hydrogen bond/salt bridge which forms between
10
11 320 the O atom of the carboxylate group and the H atom of an ammonium group in highly
12
13 321 charged region, 3) white, hydrogen bond between O atom of the carboxylate group and H
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15 322 atom on an electro-positive C atom. Residues Ala-242, Arg-396/Cys-396, Ser398, Glu-399
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17 323 (heme binding site) and His-494 (iron binding site) are represented as Connolly surface to
18
19 324 allow the visualization of the conformational changes in the TPO protein and its binding
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21 325 pocket. The Connolly surface is colored according to electrostatic potential spectrum
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23 326 (negative potential, in red, to neutral, in white, to positive, in blue). Regions in yellow rings
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25 327 highlight the interrupted hydrogen bond network observed when the wild type (i) changes to
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27 328 the mutant TPO protein (ii).
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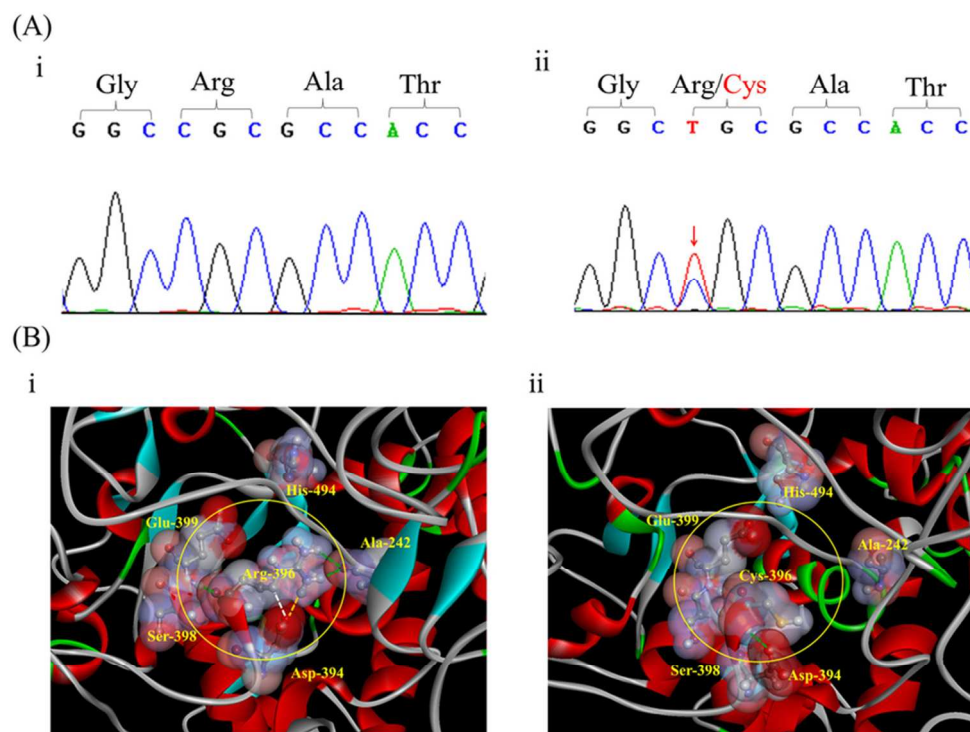


Figure 2

(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 a substitution of arginine to cysteine at codon 396 (p.Arg396Cys). (B) Homology models illustrating the 3-D orientation of the wild type (i) and mutant p.Arg396Cys (ii) 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 region, 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, Ser398, Glu-399 (heme binding site) and His-494 (iron binding site) are represented as Connolly surface to allow the visualization of the conformational changes in the TPO protein and its binding pocket. The Connolly surface is colored 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 TPO protein (ii).

78x60mm (300 x 300 DPI)

Supplementary material

Table S1: Profiles of Malaysian-Chinese patients with CH showing the respective thyroid function at the time of diagnosis. Gray shading indicates patients with the c.2268dup mutation. Reference range for: TSH, cord = < 25.0 μ IU/ml; 1 to 3 days = 2.5 - 13.0 μ IU/ml; less than a month = 0.6 - 10.0 μ IU/ml; 1 month onwards = 0.6 - 8.0 μ IU/ml and fT_4 , cord = 28.4 - 68.4 pmol/L; 1 month = 20.0 - 28.4 pmol/L; 4 months onwards = 9.0 - 24.5 pmol/L.

CH patient (CHP)	Gender	Age of diagnosis	TSH (μ IU/ml)	fT_4 (pmol/L)	Thyroid scan (at 3 years old) / ultrasonography
16	Female	10 days	26.5	11.3	Normal
18	Male	3 weeks	59.0	13.0	Multinodular goiter at 13.5 years
21	Female	3 months	20.6	14.1	Normal
24	Female	Newborn	61.3	13.6	Normal
38	Male	5 days	42.6	14.3	Normal
40	Male	4 days	27.8	18.4	Normal
45	Male	Newborn	217.0	5.0	Normal
51	Male	4 weeks	33.3	12.2	Normal
55	Male	7 weeks	>100	5.0	Normal
56	Female	5 weeks	181	5.0	Normal
58	Male	5 days	37	19.9	Normal
59	Male	3 months	>100	0.5	Normal

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Identification of two novel mutations, c.670_672del and c.1186C>T, and the prevalence of c.2268dup in the TPO gene in a cohort of Malaysian-Chinese with thyroid dyshormonogenesis

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Research**Identification of two novel mutations, c.670_672del and c.1186C>T, and the prevalence of c.2268dup in the *TPO* gene in a cohort of Malaysian-Chinese with thyroid dyshormonogenesis**

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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 dysmorphogenesis. 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 TSH and low fT_4 levels at the time of diagnosis with proven presence of thyroid gland

Primary outcome measure: Screening of the c.2268dup mutation in the *TPO* gene in all patients was carried out using 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 is a carrier for the c.2268dup mutation

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

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

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3 51 **Strengths and limitations of this study**
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- 6 52 • The c.2268dup was detected in approximately one third of the Malaysian-Chinese
7
8 53 with thyroid dysmorphogenesis. The c.2268dup in the homozygous form might be
9
10 54 associated with the phenotype of dysmorphogenetic goiter.
11
12 55 • Two novel *TPO* mutations namely c.670_672del and c.1186C>T were also detected
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14 56 in this study. *In silico* analyses revealed that the two mutations may affect the normal
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16 57 structure/function of the mutant TPO protein.
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18 58 • The *in silico* functional analyses could not be further validated due to unavailability of
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20 59 thyroid tissue samples from the patients.
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39 65 **Keywords:** Congenital hypothyroidism, *thyroid peroxidase*, c.2268dup, novel mutations,
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68 INTRODUCTION

69 Congenital hypothyroidism (CH) is one of the most common endocrine disorders in
70 the world affecting 1 in 3000 - 4000 newborn babies with 10 - 20 % of the cases are due to
71 thyroid dysmorphogenesis [1]. Over the past three decades, numerous cases of
72 dysmorphogenetic CH have been linked to alterations in the *TPO* gene [2]. This gene
73 encodes a protein with 933 amino acids in length which plays an important role in thyroid
74 hormones synthesis [3]. Niu *et al.* in 2002 reported a nonsense mutation, c.2268dup, a
75 common cause of dysmorphogenetic CH in Taiwan with molecular proof of a founder effect
76 [4]. Recently, we identified the c.2268dup mutation in a Malaysian-Chinese family with
77 goitrous CH and showed that the mutation leads to the reduction of TPO protein expression
78 with a consequential loss of enzyme activity [5]. The Chinese forms the second largest ethnic
79 group which constitutes about 24.6 % of the 28.3 million Malaysian populations [6]. As the
80 cause of dysmorphogenetic CH in Malaysian-Chinese remains unclear, we therefore
81 embarked on this present study with the aim of providing further data on the incidence of the
82 c.2268dup mutation in Malaysian Chinese and its possible phenotypic effects.

84 SUBJECTS AND METHODS

85 *Subjects*

86 A cohort (duration of follow-up between 3 and 25 years) of 12 biologically unrelated
87 Chinese patients with dysmorphogenetic CH who attended the Paediatric Endocrine Clinic,
88 University Malaya Medical Centre (UMMC) was recruited for this study. None of the
89 patients are from a consanguineous family. All patients had high TSH and low fT₄ levels at
90 the time of diagnosis with proven presence of thyroid gland (Table 1). Serum thyroglobulin
91 (hTG) level was measured in patients who have reached puberty (12 years old and older) or
92 presented with goiter; except for CHP51 and CHP55 who had been transferred to adult care

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3 93 and other hospital, respectively (Table 1). Informed written consent was obtained either from
4
5 94 patients or their parent/guardian. This study was approved by the UMMC Ethical Committee
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7 95 (Institutional Review Board) in accordance to the ICH-GCP guideline and the Declaration of
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9 96 Helsinki (Reference number, 654.16). Perchlorate discharge test was however, not performed
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11 97 in our patients since permission was not granted by the majority of the patients' parents.
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14 15 98 *TPO mutation screening*

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17 99 PCR amplification and direct DNA sequencing were performed to screen for
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19 100 alteration of *TPO* gene using genomic DNA extracted from peripheral venous blood. A
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21 101 forward (5'-ACAGGGACGTTGGTGTGTGG-3') and a reverse (5'-
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23 102 TCAGAAGCACCTTTTGGCG-3') primer were used to PCR-amplify exon 13 of the *TPO*
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25 103 gene (NM_000547.5) where the c.2268dup mutation is located. Further screening for
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27 104 mutations in other exonic regions of the *TPO* gene [7] was carried out if the patient is a
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29 105 carrier for the c.2268dup mutation. To confirm that an alteration in the *TPO* gene is due to a
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31 106 disease-causing mutation instead of a polymorphism, a total of 100 chromosomes from 50
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33 107 unrelated healthy individuals were also screened for the same mutation.
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37 38 108 *In silico analyses of the novel c.670_672del (p.Asp224del) and c.1186C>T (p.Arg396Cys)* 39 40 109 *mutations*

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43 110 The effects of the novel mutations on normal TPO activity were evaluated using SIFT
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45 111 and Polyphen-2 algorithms. Alignment of human TPO sequence with those of mouse, rat, pig,
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47 112 dog and chicken was performed using CLC Sequence Viewer 6.5.2 software (CLC bio,
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49 113 Aarhus, Denmark). The homology models of human TPO including the wild type and the two
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51 114 mutant proteins: p.Asp224del and p.Arg396Cys were generated, verified and compared as
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53 115 described before [7].
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117 RESULTS AND DISCUSSION

118 In addition to a patient with homozygous c.2268dup mutation reported in our previous
119 study [5], the c.2268dup was detected in 31 % of the total alleles studied. So far, the mutation
120 was only detected amongst patients with confirmed total iodide organification defect (TIOD)
121 tested using perchlorate discharge test [4, 8]. The test for TIOD was however, not performed
122 in our patients. This could be the reason for the higher prevalence of the c.2268dup mutation
123 in the Taiwanese patients when compared to our study [4, 8]. Nonetheless, the difference in
124 the origin between the Chinese population of Malaysia [9] and Taiwan [10] may also have
125 contributed to this variation. Two patients in this study, CHP18 and CHP59, were
126 homozygotes while another two, CHP38 and CHP58, were heterozygotes for the mutation.

127
128 Apart from the c.2268dup mutation, a novel, heterozygous c.670_672del mutation in
129 exon 7 of the *TPO* gene was detected in patient CHP58. The deletion of 3 nucleotides (GAC)
130 is predicted to produce an in-frame deletion of a single aspartic acid (p.Asp224del) in the
131 TPO protein (Fig. 1A). Three dimensional (3-D) model analysis of the mutant protein
132 revealed that the deleted Asp-224 residue is located within a beta-strand. The mutation has
133 brought conformational changes to the protein by shortening the length of the beta-strand and
134 also disrupted the correct orientation of hydrogen bonds network between Asp-223 with Asp-
135 224, Arg-225, Tyr-226 and Arg-648. Since the altered sites are located so close to His-494, a
136 proposed iron (heme axial ligand) binding site [11], it could possibly interfere with binding of
137 the iron ion at His-494 or the electron transfer activity of TPO in which His-494 is the source
138 of the electron [12]. It is also predicted that the deletion of Asp-224 will alter the orientation
139 of a salt bridge between Asp-223 and Arg-648 (Fig. 1B). Interestingly, three aspartic acid
140 residues: Asp-222, Asp-223 and Asp224 present in the same beta-strand that is located on the
141 outer surface of the wild type TPO contribute to a highly negatively-charged region which is

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3 142 also conserved across many species including mice, rat and dog implying that this region is
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5 143 crucial for the normal activity of the protein (Fig. 1C).
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7 144 Most cases of CH associated with alterations in the *TPO* gene were caused by either
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9
10 145 homozygous or compound heterozygous mutations. In the present study, three different
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12 146 alterations in the *TPO* gene were identified in CHP38 other than the confirmed
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14 147 polymorphism. In addition to the c.2268dup, a novel, heterozygous mutation, c.1186C>T
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16 148 (p.Arg396Cys), was detected in exon 8 and is expected to cause a substitution of arginine to
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18 149 cysteine at codon 396 (Fig 2A). Results from both SIFT and Polyphen-2 analyses indicated
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20 150 that the substitution is damaging and hence implying that this residue is important in the
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22 151 structure/function of the TPO. A study has shown that the Arg-396 is one of the important
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24 152 amino acids which could be involved in stabilizing the transition state of TPO protein during
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26 153 the catalytic intermediate formation [13]. The formation of a stable catalytic intermediate
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28 154 (compound I) of the TPO with H₂O₂ is crucial for thyroid hormone synthesis [14-15].
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30 155 Catalytic process is initiated by the diffusion of H₂O₂ into the active site of the TPO protein.
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32 156 The α -nucleophile H₂O₂ donates a proton to the distal imidazole ring (His-239) to form a
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34 157 bond with the iron ion bound to residue His-494. After binding takes place, the protein attains
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36 158 transition state to form compound I. The arginine at position 396 is believed to play a role in
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38 159 stabilizing the charge for transition state of the protein through electrostatic interaction
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40 160 [13,16]. Alternatively, it is believed that the arginine contributes to the abnormally low pKa
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42 161 value of the distal histidine in the native resting enzyme. The changes of the pKa value in the
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44 162 transition state of the distal imidazole are the key to determine the effectiveness of the
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46 163 catalysis process/rate of the compound I formation [16]. Therefore, a substitution from
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48 164 arginine to cysteine can bring devastating effects to the protein stability. In the present study,
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50 165 the 3-D model analysis showed that the p.Arg396Cys mutation has led to the structure
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3 166 alteration through the modification of the hydrogen bond network in the hydrophobic pocket
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5 167 which might interfere with the heme binding at Glu-399 [17] (Fig. 2B).
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7 168 Apart from the c.2268dup and c.1186C>T mutations, a non-synonymous substitution,
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9 169 c.2647C>T, was also identified in exon 16 of CHP38. The nucleotide alteration leads to a
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11 170 substitution of proline to serine at codon 883 in the C-terminal tail (Val-869 to Leu-933) of
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13 171 the TPO protein and was reported in patient with dysmorphogenetic CH in populations of
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15 172 Korea [18] and Japan [19] before. However, the consequence of the c.2647C>T remains
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17 173 ambiguous since both SIFT and Polyphen-2 analyses result [2] indicates that this alteration is
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19 174 “benign” suggesting that the Pro-883 is probably not critical for structure/function of the TPO
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21 175 and could be a rare polymorphism instead of disease-causing allele.
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27 177 It is worth noting that CHP18 and the two sisters reported in our previous study [5]
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29 178 with homozygous c.2268dup had developed large multinodular goiters in their mid or late
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31 179 adolescent years. Although it is not known whether the reduction in TPO expression due to
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33 180 c.2268dup can lead to increased risk of malignant transformation, other studies have shown
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35 181 cases of thyroid carcinoma has developed from congenital goiters that are associated with
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37 182 *TPO* mutation [20-21] or with lower/absence of TPO expression [22-23]. Therefore, it is
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39 183 important to have a careful surveillance for potential thyroid neoplasm in patients with
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41 184 c.2268dup mutation.
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47 186 **CONCLUSION**

48
49 187 In conclusion, we report two novel mutations in the *TPO* gene, c.670_672del and
50
51 188 c.1186C>T, and reveal the association of c.2268dup mutation with approximately one third
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53 189 of a cohort of Malaysian-Chinese with dysmorphogenetic CH. This study also support our
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55 190 previous findings that c.2268dup homozygotes developed dysmorphogenetic goiter in their
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191 mid or late adolescent years. These data will be useful in diagnosing or predicting goitrous
192 dysshormonogenetic CH.
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3 194 **Contributors:**

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5 195 CCL participated in research design, performed the experiments, analyzed the data and wrote
6
7 196 the paper; FH and MYJ collected clinical samples and analyzed the clinical data; CHH and
8
9 197 RO performed the 3D analyses and interpreted the data; SMJ proposed research design,
10
11 198 analyzed the molecular and overall data, and participated in writing the paper.
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15
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19
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21
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26 204 **Competing interests:** None
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30 206 **Ethics approval:** The University of Malaya Medical Centre (UMMC) Ethical Committee
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35 208 **Data sharing statement:** No additional data are available
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211 **FIGURE-LEGEND**

212 **Figure 1:** (A) DNA sequencing profiles. Electropherogram profiles of a control with a wild
213 type allele (i), and CHP51 who is a heterozygote for the c.670_672del mutation (ii). The three
214 deleted nucleotides (GAC) are indicated by the arrows. The sequence alteration is predicted
215 to produce an in-frame deletion of a single amino acid, aspartic acid (p.Asp224del). (B)
216 Homology models illustrating the 3-D orientation of the wild type (i) and mutant
217 p.Asp224del (ii) TPO proteins. The protein backbones are presented as ribbons (alpha-helix
218 in red, beta-pleated sheet in cyan, coils in grey, and turns in green). Hydrogen bonds are
219 highlighted in: 1) green, hydrogen bond under the normal criteria, 2) brown, hydrogen
220 bond/salt bridge which forms between the O atom of the carboxylate group and the H atom of
221 an ammonium group in highly charged region. Residues Arg-223 to Tyr-226, Arg-648 and
222 His-494 (iron binding site) are represented as Connolly surface to allow the visualization of
223 the conformational changes in the TPO protein and its binding pocket. The Connolly surface
224 is colored according to electrostatic potential spectrum (negative potential, in red, to neutral,
225 in white, to positive, in blue). Regions in yellow rings highlight the interrupted hydrogen
226 bond network observed when the wild type (i) changes to the mutant TPO protein (ii).
227 (C) Multiple-sequence alignment of human TPO with those of mouse, rat, pig, dog and
228 chicken. The alignment data show that the negatively charge region (Asp-222, Asp-223 and
229 Asp224) is conserved amongst human and many different animal species. The position of the
230 deleted residue (p.Asp224del) is indicated by the arrow.

231 **Figure 2**

232 (A) DNA sequencing profiles. Electropherogram profiles of a control with a wild type allele
233 (i), and CHP38 who is a heterozygote for the c.1186C>T mutation (ii). The single nucleotide
234 transition is indicated by the arrow. The sequence alteration is predicted to cause a
235 substitution of arginine to cysteine at codon 396 (p.Arg396Cys). (B) Homology models

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3 236 illustrating the 3-D orientation of the wild type (i) and mutant p.Arg396Cys (ii) TPO proteins.
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5 237 The protein backbones are presented as ribbons (alpha-helix in red, beta-pleated sheet in cyan,
6
7 238 coils in grey, and turns in green). Hydrogen bonds are highlighted in: 1) green, hydrogen
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9 239 bond under the normal criteria, 2) brown, hydrogen bond/salt bridge which forms between
10
11 240 the O atom of the carboxylate group and the H atom of an ammonium group in highly
12
13 241 charged region, 3) white, hydrogen bond between O atom of the carboxylate group and H
14
15 242 atom on an electro-positive C atom. Residues Ala-242, Arg-396/Cys-396, Ser398, Glu-399
16
17 243 (heme binding site) and His-494 (iron binding site) are represented as Connolly surface to
18
19 244 allow the visualization of the conformational changes in the TPO protein and its binding
20
21 245 pocket. The Connolly surface is colored according to electrostatic potential spectrum
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23 246 (negative potential, in red, to neutral, in white, to positive, in blue). Regions in yellow rings
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25 247 highlight the interrupted hydrogen bond network observed when the wild type (i) changes to
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27 248 the mutant TPO protein (ii).
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36 **Table 1:** Profiles of Malaysian-Chinese patients with CH showing the respective thyroid
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38 251 function at the time of diagnosis. Gray shading indicates patients with the c.2268dup
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40 252 mutation. Reference range for: TSH, cord = < 25.0 μ IU/ml; 1 to 3 days = 2.5 - 13.0 μ IU/ml;
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42 253 less than a month = 0.6 - 10.0 μ IU/ml; 1 month onwards = 0.6 - 8.0 μ IU/ml, fT₄, cord = 28.4
43
44 254 - 68.4 pmol/L; 1 month = 20.0 - 28.4 pmol/L; 4 months onwards = 9.0 - 24.5 pmol/L and
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46 255 hTG, (0-55.0 ng/ml). N/A: hTG level was not measured in patients below 12 years of age,
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48 256 unless goiter is present. CHP51 and CHP55 were transferred to adult care and other hospital
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Table 1: Profiles of Malaysian-Chinese patients with CH showing the respective thyroid function at the time of diagnosis. Gray shading indicates patients with the c.2268dup mutation. Reference range for: TSH, cord = < 25.0 μ IU/ml; 1 to 3 days = 2.5 - 13.0 μ IU/ml; less than a month = 0.6 - 10.0 μ IU/ml; 1 month onwards = 0.6 - 8.0 μ IU/ml, fT₄, cord = 28.4 - 68.4 pmol/L; 1 month = 20.0 - 28.4 pmol/L; 4 months onwards = 9.0 - 24.5 pmol/L and hTG, (0-55.0 ng/ml). N/A: hTG level was not measured in patients younger than 12 years of age, unless goiter was present. CHP51 and CHP55 were transferred to adult care and other hospital respectively.

CH patient (CHP)	Gender	Age of diagnosis	Duration of follow-up (years)	TSH (μ IU/ml)	fT ₄ (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 goiter 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

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338	59	Male	3 months	15	>100	0.5	283	Normal
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Research

Identification of two novel mutations, c.670_672del and c.1186C>T, and the prevalence of c.2268dup in the *TPO* gene in a cohort of Malaysian-Chinese with thyroid dyshormonogenesis

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ABSTRACT

Objectives: The c.2268dup mutation in the thyroid peroxidase (*TPO*) gene is the most common *TPO* ~~defect~~alteration reported in Taiwanese patients with thyroid dysmorphogenesis. 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 ~~*TPO* gene~~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 TSH and low fT₄ levels at the time of diagnosis with proven presence of thyroid gland

Primary outcome measure: Screening of the c.2268dup mutation in the *TPO* gene in all patients was carried out using 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 is a carrier for the c.2268dup mutation

Results: The c.2268dup mutation was detected in 4 out of the 12 patients. ~~A homozygous patient, CHP59 had developed large multinodular goiter at the age of 13.5 years which supports our previous findings that c.2268dup homozygotes developed dysmorphogenetic goiter in their mid or late adolescent years. Apart from the c.2268dup and a~~ Apart from the c.2268dup and a previously documented mutation, c.2647C>T, two novel *TPO* mutations namely c.670_672del and c.1186C>T were also detected in our patients. *In silico* analyses predicted that the novel mutations affect the structure/function of the TPO protein.

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7 50 **Conclusion:** The c.2268dup was detected in approximately one third of the Malaysian-
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9 51 Chinese with thyroid dysmorphogenesis. The detection of the novel c.670_672del and
10 52 c.1186C>T alterations expand the mutation spectrum of *TPO* associated with thyroid
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12 53 dysmorphogenesis.
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14 54

16 55 **Strengths and limitations of this study**

- 19 56 • The c.2268dup was detected in approximately one third of the Malaysian-Chinese
20 57 with thyroid dysmorphogenesis. The c.2268dup in the homozygous form might be
21 58 associated with the phenotype of dysmorphogenetic goiter.
- 25 59 • Two novel *TPO* mutations namely c.670_672del and c.1186C>T were also detected
26 60 in this study. *In silico* analyses revealed that the two mutations may affect the normal
27 61 structure/function of the mutant *TPO* protein.
- 30 62 • The *in silico* functional analyses could not be further validated due to unavailability of
31 63 thyroid tissue samples from the patients.
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48 69 **Keywords:** Congenital hypothyroidism, *thyroid peroxidase*, c.2268dup, novel mutations,
49 70 Malaysian-Chinese
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72 INTRODUCTION

73 Congenital hypothyroidism (CH) is one of the most common endocrine disorders in
 74 the world affecting 1 in 3000 - 4000 newborn babies ~~where~~with 10 - 20 % of the cases
 75 ~~were~~are due to thyroid dysmorphogenesis [1]. Over the past three decades, numerous cases
 76 of dysmorphogenetic CH have been linked to ~~defects~~alterations in the *TPO* gene [2]. This
 77 gene encodes a protein with 933 amino acids in length which plays an important role in
 78 thyroid hormones synthesis [3]. Niu *et al.* in 2002 reported a nonsense mutation, c.2268dup, a
 79 common cause of dysmorphogenetic CH in Taiwan with molecular proof of a founder effect
 80 ~~where the ancestors of these patients originated from the southern province of China [4].~~[4].
 81 Recently, we identified the c.2268dup mutation in a Malaysian-Chinese family with goitrous
 82 CH and showed that the mutation leads to the reduction of TPO protein expression with a
 83 consequential loss of enzyme activity [5]. The Chinese forms the second largest ethnic group
 84 which constitutes about 24.6 % of the 28.3 million Malaysian populations [6]. As the cause of
 85 dysmorphogenetic CH in Malaysian-Chinese remains unclear, we therefore embarked on
 86 this present study with the aim of providing further data on the incidence of ~~TPO gene~~the
 87 c.2268dup mutation in Malaysian Chinese and its possible phenotypic effects.

89 SUBJECTS AND METHODS

90 ~~Subjects for the TPO mutation screening~~

91 A cohort (duration of follow-up between 3 and 25 years) of 12 biologically unrelated
 92 Chinese patients with dysmorphogenetic CH who attended the Paediatric Endocrine Clinic,
 93 University Malaya Medical Centre (UMMC) was recruited for this study. ~~Mutational~~
 94 ~~screening was performed on genomic DNA extracted from peripheral venous blood of all~~
 95 ~~patients who~~None of the patients are from a consanguineous family. All patients had high
 96 TSH and low fT₄ levels at the time of diagnosis with proven presence of thyroid gland (Table

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7 97 1). Serum thyroglobulin (hTG) level was measured in patients who have reached puberty (12
8 years old and older) or presented with goiter; except for CHP51 and CHP55 who had been
9 transferred to adult care and other hospital, respectively (Table 1). Informed written consent
10 was obtained either from patients or their parent/guardian. This study was approved by the
11 UMMC Ethical Committee (Institutional Review Board) in accordance to the ICH-GCP
12 guideline and the Declaration of Helsinki (Reference number, 654.16). Perchlorate discharge
13 test was however, not performed in our patients since permission was not granted by the
14 majority of the patients' parents.

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23 105 TPO mutation screening^{S1}):

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25 106 PCR amplification and direct DNA sequencing were performed to screen for
26 alteration of TPO gene using genomic DNA extracted from peripheral venous blood. A

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27 107 forward (5'-ACAGGGACGTTGGTGTGTGG-3') and a reverse (5'-
28 TCAGAAGCACCTTTTGCG-3') primer were used to PCR-amplify exon 13 of the TPO
29 gene (NM_000547.5) where the c.2268dup mutation is located. Further screening for
30 mutations in other exonic regions of the TPO gene [7] was carried out if the patient is a
31 carrier for the c.2268dup mutation. To confirm that an alteration in the TPO gene is due to a
32 disease-causing mutation instead of a polymorphism, a total of 100 chromosomes from 50
33 unrelated healthy individuals were also screened for the same mutation. Informed written

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40 114 ~~consent was obtained either from patients or their parent/guardian. This study was approved~~
41 ~~by the UMMC Ethical Committee (Institutional Review Board) in accordance to the ICH-~~
42 ~~GCP guideline and the Declaration of Helsinki (Reference number, 654.16).~~

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48 118 *In silico analyses of the novel c.670_672del (p.Asp224del) and c.1186C>T (p.Arg396Cys)*
49 *mutations*
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7 120 The effects of the novel mutations on normal TPO activity were evaluated using SIFT
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9 121 and Polyphen-2 algorithms. Alignment of human TPO sequence with those of mouse, rat, pig,
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11 122 dog and chicken was performed using CLC Sequence Viewer 6.5.2 software (CLC bio,
12
13 123 Aarhus, Denmark). The homology models of human TPO including the wild type and the two
14
15 124 mutant proteins: p.Asp224del and p.Arg396Cys were generated, verified and compared as
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17 125 described before [7].
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21 127 RESULTS AND DISCUSSION

24 128 ~~The In addition to a patient with homozygous c.2268dup mutation was detected in 4~~
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26 129 ~~out of the total 12 unrelated patients in this study. Two patients, CHP18 and CHP59, were~~
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28 130 ~~homozygotes while another two, CHP38 and CHP58, were heterozygotes for the mutation. In~~
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30 131 ~~addition to a case reported in our previous study [5], the c.2268dup was detected in only~~
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32 132 ~~31 % of the total alleles studied, lower than what were reported in the studies by Niu *et al.*~~
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34 133 ~~(2002) (86 %) [4] and Wu *et al.* (2002) (40 %) [8]. For their study, only. So far, the mutation~~
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36 134 ~~was only detected amongst patients with confirmed total iodide organification defect (TIOD)~~
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38 135 ~~through tested using perchlorate discharge test were screened for the TPO gene mutations. In~~
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40 136 ~~contrast, the perchlorate [4, 8]. The test for TIOD was however, not performed in our patients~~
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42 137 ~~since permission was not granted by the majority of the patients' parents. This could be the~~
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44 138 ~~reason for the higher prevalence of the c.2268dup mutation in the Taiwanese patients when~~
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46 139 ~~compared to our study [4, 8]. Nonetheless, the difference in the origin between the Chinese~~
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48 140 ~~population of Malaysia [9] and Taiwan [10] may also have contributed to this variation. Two~~
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50 141 ~~patients in this study, CHP18 and CHP59, were homozygotes while another two, CHP38 and~~
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52 142 ~~CHP58, were heterozygotes for the mutation.~~

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7 144 | Apart from the c.2268dup mutation, a novel, heterozygous c.670_672del mutation in
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9 145 | exon 7 of the *TPO* gene was detected in patient CHP58. The deletion of 3 nucleotides (GAC)
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11 146 | is predicted to produce an in-frame deletion of a single aspartic acid (p.Asp224del) in the
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13 147 | *TPO* protein (Fig. 1A). Three dimensional (3-D) model analysis of the mutant protein
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15 148 | revealed that the deleted Asp-224 residue is located within a beta-strand. The mutation has
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17 149 | brought conformational changes to the protein by shortening the length of the beta-strand and
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19 150 | also disrupted the correct orientation of hydrogen bonds network between Asp-223 with Asp-
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21 151 | 224, Arg-225, Tyr-226 and Arg-648. Since the altered sites are located so close to His-494, a
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23 152 | proposed iron (heme axial ligand) binding site [11], it could possibly interfere with binding of
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25 153 | the iron ion at His-494 or the electron transfer activity of *TPO* wherein which His-494 is the
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27 154 | source of the electron [12]. It is also predicted that the deletion of Asp-224 will alter the
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29 155 | orientation of a salt bridge between Asp-223 and Arg-648 (Fig. 1B). Interestingly, three
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31 156 | aspartic acid residues: Asp-222, Asp-223 and Asp224 present in the same beta-strand that is
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33 157 | located on the outer surface of the wild type *TPO* contribute to a highly negatively-charged
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35 158 | region which is also conserved across many species including mice, rat and dog implying that
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37 159 | this region is crucial for the normal activity of the protein (Fig. 1C).

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38 160 | Most cases of CH associated with defects/alterations in the *TPO* gene were caused by
39
40 161 | either homozygous or compound heterozygous mutations. In the present study, three different
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42 162 | mutations/alterations in the *TPO* gene were identified in CHP38- other than the confirmed
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44 163 | polymorphism. In addition to the c.2268dup, a novel, heterozygous mutation, c.1186C>T
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46 164 | (p.Arg396Cys), was detected in exon 8 and is expected to cause a substitution of arginine to
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48 165 | cysteine at codon 396 (Fig 2A). Results from both SIFT and Polyphen-2 analyses indicated
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50 166 | that the substitution is damaging and hence implying that this residue is important in the
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52 167 | structure/function of the *TPO*. A study has shown that the Arg-396 is one of the important
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54 168 | amino acids which could be involved in stabilizing the transition state of *TPO* protein during

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7 169 the catalytic intermediate formation [13]. The formation of a stable catalytic intermediate
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9 170 (compound I) of the TPO with H₂O₂ is crucial for thyroid hormone synthesis [14-15].
10
11 171 Catalytic process is initiated by the diffusion of H₂O₂ into the active site of the TPO protein.
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13 172 The α -nucleophile H₂O₂ donates a proton to the distal imidazole ring (His-239) to form a
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15 173 bond with the iron ion bound to residue His-494. After binding takes place, the protein attains
16
17 174 transition state to form compound I. The arginine at position 396 is believed to play a role in
18
19 175 stabilizing the charge for transition state of the protein through electrostatic interaction
20
21 176 [13,16]. Alternatively, it is believed that the arginine contributes to the abnormally low pKa
22
23 177 value of the distal histidine in the native resting enzyme. The changes of the pKa value in the
24
25 178 transition state of the distal imidazole are the key to determine the effectiveness of the
26
27 179 catalysis process/rate of the compound I formation [16]. Therefore, a substitution from
28
29 180 arginine to cysteine can bring devastating effects to the protein stability. In the present study,
30
31 181 the 3-D model analysis showed that the p.Arg396Cys mutation has led to the structure
32
33 182 alteration through the modification of the hydrogen bond network in the hydrophobic pocket
34
35 183 which might interfere with the heme binding at Glu-399 [17] (Fig. 2B).

36 184 ~~— Apart from the c.2268dup and c.1186C>T mutations, a non-synonymous~~
37 185 ~~substitution, c.2647C>T mutation, was also identified in exon 16 of CHP38, where it. The~~
38 186 ~~nucleotide alteration leads to a substitution of proline to serine at codon 883 in the C-terminal~~
39 187 ~~tail (Val-869 to Leu-933) of the TPO protein, and was reported in patient with~~
40 188 ~~dyshormonogenetic CH in populations of Korea [18] and Japan [19] before. However, the~~
41 189 ~~consequence of the c.2647C>T remains ambiguous since both SIFT and Polyphen-2 analyses~~
42 190 ~~result from a previous study [2] indicates that this mutational alteration is “benign” suggesting~~
43 191 ~~that the Pro-883 is probably not critical for structure/function of the TPO and does not~~
44 192 ~~modify TPO function. Interestingly, this mutation had also been reported in populations of~~
45 193 ~~Korea [18] and Japan [19]. Whether the c.2647C>T mutation is possibly a rare polymorphism~~

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7 194 ~~is not known. Despite the *in silico* findings indicated that the substitution of Pro 883 to serine~~
8 195 ~~might not be critical to the protein structure/function, previously reported cases showed that~~
9 196 ~~patients associated with this mutation presented with severe dysmorphogenetic CH [18-19].~~
10 197 ~~Since Human Splicing Finder (HSF) analysis predicted that the c.2647C>T mutation~~
11 198 ~~interrupts the sequences recognized by ESE proteins (data not shown), we therefore could not~~
12 199 ~~exclude that the sequence alteration might possibly regulate the splicing activity of the TPO~~
13 200 ~~pre-mRNA and lead to CH in these patients. could be a rare polymorphism instead of disease-~~
14 201 ~~causing allele.~~

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24 203 It is worth noting that ~~patients with homozygous c.2268dup inclusive of CHP18 and~~
25 204 ~~the two patients/sisters~~ reported in our previous study [5], ~~except for patient CHP59 who was~~
26 205 ~~12-year-old at the time of the study,] with homozygous c.2268dup~~ had developed large
27 206 multinodular ~~goiter/goiters~~ in their mid or late adolescent years. Although it is not known
28 207 whether the reduction in TPO expression due to c.2268dup can lead to increased risk of
29 208 malignant transformation, other studies have shown cases of thyroid carcinoma has
30 209 developed from congenital ~~goiter/goiters~~ that ~~is/are~~ associated with *TPO* mutation [20-21] or
31 210 ~~with~~ lower/-absence of TPO expression [22-23]. Therefore, it is important to have a careful
32 211 surveillance for potential thyroid neoplasm in patients with c.2268dup mutation.

212 213 CONCLUSION

214 In conclusion, we report two novel mutations in the *TPO* gene, c.670_672del and
215 c.1186C>T, and reveal the association of c.2268dup mutation with approximately one third
216 of a cohort of Malaysian-Chinese with dysmorphogenetic CH. This study also support our
217 previous findings that c.2268dup homozygotes developed dysmorphogenetic goiter in their

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218 mid or late adolescent years. These data will be useful in diagnosing or predicting goitrous
219 dysshormonogenetic CH.
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221 **Contributors:**

222 CCL participated in research design, performed the experiments, analyzed the data and wrote
 223 the paper; FH and MYJ collected clinical samples and analyzed the clinical data; CHH and
 224 RO performed the 3D analyses and interpreted the data; SMJ proposed research design,
 225 analyzed the molecular and overall data, and participated in writing the paper.

227 **Funding:**

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 229 Malaysia (~~H-20001-00-E000009 and FP050/2010B~~) and ~~Postgraduate Research Fund,~~
 230 ~~University of Malaya (PV116-2012A), and FP034-2014A).~~

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232 **Competing interests:** None

234 **Ethics approval:** The University of Malaya Medical Centre (UMMC) Ethical Committee

236 **Data sharing statement:** No additional data are available

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7 313 **FIGURE-LEGEND**

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9 314 **Figure 1:** (A) DNA sequencing profiles. Electropherogram profiles of a control with a wild
10 315 type allele (i), and CHP51 who is a heterozygote for the c.670_672del mutation (ii). The three
11 316 deleted nucleotides (GAC) are indicated by the arrows. The sequence alteration is predicted
12 317 to produce an in-frame deletion of a single amino acid, aspartic acid (p.Asp224del). (B)
13 318 Homology models illustrating the 3-D orientation of the wild type (i) and mutant
14 319 p.Asp224del (ii) TPO proteins. The protein backbones are presented as ribbons (alpha-helix
15 320 in red, beta-pleated sheet in cyan, coils in grey, and turns in green). Hydrogen bonds are
16 321 highlighted in: 1) green, hydrogen bond under the normal criteria, 2) brown, hydrogen
17 322 bond/salt bridge which forms between the O atom of the carboxylate group and the H atom of
18 323 an ammonium group in highly charged region. Residues Arg-223 to Tyr-226, Arg-648 and
19 324 His-494 (iron binding site) are represented as Connolly surface to allow the visualization of
20 325 the conformational changes in the TPO protein and its binding pocket. The Connolly surface
21 326 is colored according to electrostatic potential spectrum (negative potential, in red, to neutral,
22 327 in white, to positive, in blue). Regions in yellow rings highlight the interrupted hydrogen
23 328 bond network observed when the wild type (i) changes to the mutant TPO protein (ii).
24 329 (C) Multiple-sequence alignment of human TPO with those of mouse, rat, pig, dog and
25 330 chicken. The alignment data show that the negatively charge region (Asp-222, Asp-223 and
26 331 Asp224) is conserved amongst human and many different animal species. The position of the
27 332 deleted residue (p.Asp224del) is indicated by the arrow.

28 333 **Figure 2**

29 334 (A) DNA sequencing profiles. Electropherogram profiles of a control with a wild type allele
30 335 (i), and CHP38 who is a heterozygote for the c.1186C>T mutation (ii). The single nucleotide
31 336 transition is indicated by the arrow. The sequence alteration is predicted to cause a
32 337 substitution of arginine to cysteine at codon 396 (p.Arg396Cys). (B) Homology models

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7 338 illustrating the 3-D orientation of the wild type (i) and mutant p.Arg396Cys (ii) TPO proteins.
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9 339 The protein backbones are presented as ribbons (alpha-helix in red, beta-pleated sheet in cyan,
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11 340 coils in grey, and turns in green). Hydrogen bonds are highlighted in: 1) green, hydrogen
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13 341 bond under the normal criteria, 2) brown, hydrogen bond/salt bridge which forms between
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15 342 the O atom of the carboxylate group and the H atom of an ammonium group in highly
16
17 343 charged region, 3) white, hydrogen bond between O atom of the carboxylate group and H
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19 344 atom on an electro-positive C atom. Residues Ala-242, Arg-396/Cys-396, Ser398, Glu-399
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21 345 (heme binding site) and His-494 (iron binding site) are represented as Connolly surface to
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23 346 allow the visualization of the conformational changes in the TPO protein and its binding
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25 347 pocket. The Connolly surface is colored according to electrostatic potential spectrum
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27 348 (negative potential, in red, to neutral, in white, to positive, in blue). Regions in yellow rings
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29 349 highlight the interrupted hydrogen bond network observed when the wild type (i) changes to
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31 350 the mutant TPO protein (ii).

32 351
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35 352 Table 1: Profiles of Malaysian-Chinese patients with CH showing the respective thyroid
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37 353 function at the time of diagnosis. Gray shading indicates patients with the c.2268dup
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39 354 mutation. Reference range for: TSH, cord = < 25.0 µIU/ml; 1 to 3 days = 2.5 - 13.0 µIU/ml;
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41 355 less than a month = 0.6 - 10.0 µIU/ml; 1 month onwards = 0.6 - 8.0 µIU/ml, fT₄, cord = 28.4
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43 356 - 68.4 pmol/L; 1 month = 20.0 - 28.4 pmol/L; 4 months onwards = 9.0 - 24.5 pmol/L and
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45 357 hTG, (0-55.0 ng/ml). N/A: hTG level was not measured in patients below 12 years of age,
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47 358 unless goiter is present. CHP51 and CHP55 were transferred to adult care and other hospital
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49 359 respectively.

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Table 1: Profiles of Malaysian-Chinese patients with CH showing the respective thyroid function at the time of diagnosis. Gray shading indicates patients with the c.2268dup mutation. Reference range for: TSH, cord = < 25.0 μ IU/ml; 1 to 3 days = 2.5 - 13.0 μ IU/ml; less than a month = 0.6 - 10.0 μ IU/ml; 1 month onwards = 0.6 - 8.0 μ IU/ml, fT₄, cord = 28.4 - 68.4 pmol/L; 1 month = 20.0 - 28.4 pmol/L; 4 months onwards = 9.0 - 24.5 pmol/L and hTG, (0-55.0 ng/ml). N/A: hTG level was not measured in patients younger than 12 years of age, unless goiter was present. CHP51 and CHP55 were transferred to adult care and other hospital respectively.

CH patient (CHP)	Gender	Age of diagnosis	Duration of follow-up (years)	TSH (μ IU/ml)	fT ₄ (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 goiter 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

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59	Male	3 months	15	>100	0.5	283	Normal
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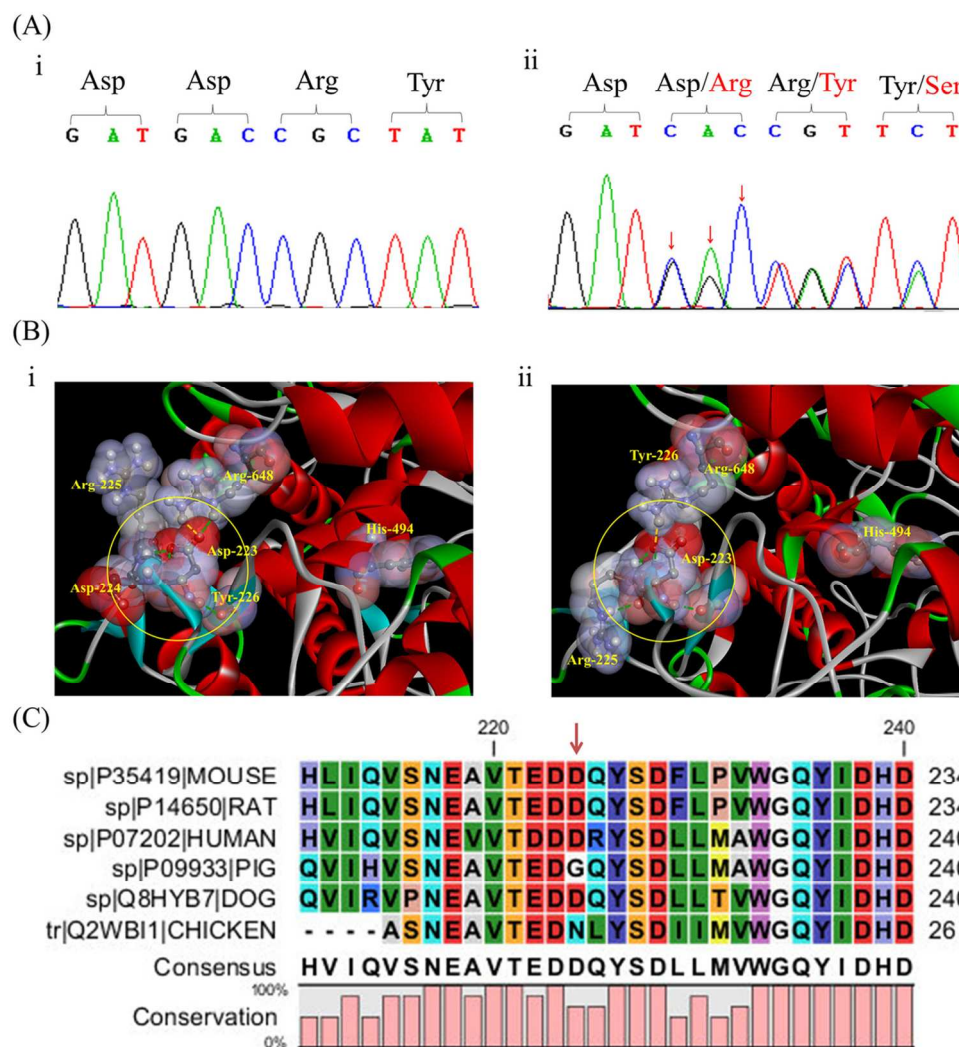


Figure 1: (A) DNA sequencing profiles. Electropherogram profiles of a control with a wild type allele (i), and CHP51 who is a heterozygote for the c.670_672del mutation (ii). The three deleted nucleotides (GAC) are indicated by the arrows. The sequence alteration is predicted to produce an in-frame deletion of a single amino acid, aspartic acid (p.Asp224del). (B) Homology models illustrating the 3-D orientation of the wild type (i) and mutant p.Asp224del (ii) 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 region. Residues Arg-223 to Tyr-226, Arg-648 and His-494 (iron binding site) are represented as Connolly surface to allow the visualization of the conformational changes in the TPO protein and its binding pocket. The Connolly surface is colored 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 TPO protein (ii). (C) Multiple-sequence alignment of human TPO with those of mouse, rat, pig, dog and chicken. The alignment data show that the negatively charge region (Asp-222, Asp-223 and Asp224) is conserved amongst human and many different animal species. The position of the deleted residue (p.Asp224del) is indicated by the arrow.

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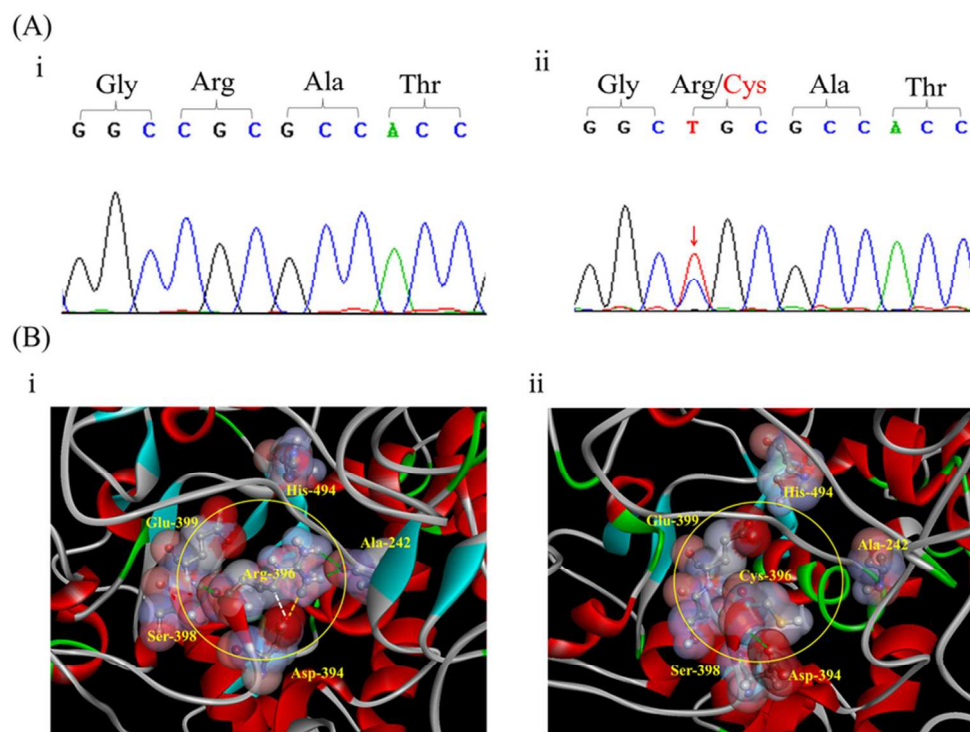


Figure 2

(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 a substitution of arginine to cysteine at codon 396 (p.Arg396Cys). (B) Homology models illustrating the 3-D orientation of the wild type (i) and mutant p.Arg396Cys (ii) 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 region, 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, Ser398, Glu-399 (heme binding site) and His-494 (iron binding site) are represented as Connolly surface to allow the visualization of the conformational changes in the TPO protein and its binding pocket. The Connolly surface is colored 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 TPO protein (ii).

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BMJ Open

The 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 dyshormonogenesis

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The 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 dyshormonogenesis

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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 dysmorphogenesis. 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 TSH and low fT₄ levels at the time of diagnosis with proven presence of thyroid gland

Primary outcome measure: Screening of the c.2268dup mutation in the *TPO* gene in all patients was carried out using 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 is a carrier for the c.2268dup mutation

Results: The c.2268dup mutation was detected in 4 out of the 12 patients. Apart from the c.2268dup and a previously documented mutation, c.2647C>T, two novel *TPO* alterations namely 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.

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

Strengths and limitations of this study

- The c.2268dup was detected in approximately one third of the Malaysian-Chinese with thyroid dyshormonogenesis. The c.2268dup in the homozygous form might be associated with the phenotype of dyshormonogenetic goiter.
- Two novel *TPO* alterations namely 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 due to unavailability of thyroid tissue samples from the patients.

Keywords: Congenital hypothyroidism, *thyroid peroxidase*, c.2268dup, novel mutations, Malaysian-Chinese

INTRODUCTION

Congenital hypothyroidism (CH) is one of the most common endocrine disorders in the world affecting 1 in 3000 - 4000 newborn babies with 10 - 20 % of the cases are due to thyroid dyshormonogenesis [1]. Over the past three decades, numerous cases of dyshormonogenetic CH have been linked to alterations in the *TPO* gene [2-3]. This gene encodes a protein with 933 amino acids in length which plays an important role in thyroid hormones synthesis [4]. Niu *et al.* in 2002 reported a nonsense mutation, c.2268dup, a common cause of dyshormonogenetic CH in Taiwan with molecular proof of a founder effect [5]. Recently, we identified the c.2268dup mutation in a Malaysian-Chinese family with goitrous CH and showed that the mutation leads to the reduction of TPO protein expression with a consequential loss of enzyme activity [6]. The Chinese forms the second largest ethnic group which constitutes about 24.6 % of the 28.3 million Malaysian populations [7]. As the cause of dyshormonogenetic CH in Malaysian-Chinese remains unclear, we therefore embarked on this present 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 between 3 and 25 years) of 12 biologically unrelated Chinese patients with dyshormonogenetic CH who attended the Paediatric Endocrine Clinic, University Malaya Medical Centre (UMMC) was recruited for this study. None of the patients are from a consanguineous family. All patients had high TSH and low fT₄ levels at the time of diagnosis with proven presence of thyroid gland (Table 1). Serum thyroglobulin (hTG) level was measured in patients who have reached puberty (12 years old and older) or presented with goiter; except for CHP51 and CHP55 who had been transferred to adult care and other

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2
3 hospital, respectively (Table 1). Informed written consent was obtained either from patients or
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5 their parent/guardian. This study was approved by the UMMC Ethical Committee
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7 (Institutional Review Board) in accordance to the ICH-GCP guideline and the Declaration of
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9 Helsinki (Reference number, 654.16). Perchlorate discharge test was however, not performed
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11 in our patients since permission was not granted by the majority of the patients' parents.
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14 *TPO mutation screening*

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17 PCR amplification and direct DNA sequencing were performed to screen for alteration
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19 of *TPO* gene using genomic DNA extracted from peripheral venous blood. A forward (5'-
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21 ACAGGGACGTTGGTGTGTGG-3') and a reverse (5'-TCAGAAGCACCTTTTGGCG-3')
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23 primer were used to PCR-amplify exon 13 of the *TPO* gene (NM_000547.5) where the
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25 c.2268dup mutation is located. Further screening for mutations in other exonic regions of the
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27 *TPO* gene [8] was carried out if the patient is a carrier for the c.2268dup mutation. To confirm
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29 that an alteration in the *TPO* gene is due to a disease-causing mutation instead of a
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31 polymorphism, a total of 100 chromosomes from 50 unrelated healthy individuals were also
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33 screened for the same mutation.
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36 37 *In silico analyses of the novel c.670_672del (p.Asp224del) and c.1186C>T (p.Arg396Cys)* 38 39 40 *mutations*

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43 The effects of the novel mutations on normal TPO activity were evaluated using SIFT
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45 and Polyphen-2 algorithms. Alignment of human TPO sequence with those of mouse, rat, pig,
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47 dog and chicken was performed using CLC Sequence Viewer 6.5.2 software (CLC bio,
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49 Aarhus, Denmark). The homology models of human TPO including the wild type and the two
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51 mutant proteins: p.Asp224del and p.Arg396Cys were generated, verified and compared as
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53 described before [8].
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RESULTS AND DISCUSSION

In addition to a patient with homozygous c.2268dup mutation reported in our previous study [6], the c.2268dup was detected in 31 % of the total alleles studied. So far, the mutation was only detected amongst patients with confirmed total iodide organification defect (TIOD) tested using perchlorate discharge test [5, 9]. 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 when compared to our study [5, 9]. Nonetheless, the difference in the origin between the Chinese population of Malaysia [10] and Taiwan [11] may also have contributed to this variation. Further studies on the c.2268dup mutation by increasing sample size and collecting information on the ancestral origins of the patients is expected to gain a deeper understanding of the frequency and distribution pattern of the c.2268dup mutation in 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 3 nucleotides (GAC) is predicted to produce an in-frame deletion of a single aspartic acid (p.Asp224del) in the TPO protein (Fig. 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 brought conformational changes to the protein by shortening the length of the beta-strand and also disrupted the correct orientation of hydrogen bonds 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 (heme axial ligand) binding site [12], 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 [13]. It is also predicted that the deletion of Asp-224 will alter the orientation

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3 of a salt bridge between Asp-223 and Arg-648 (Fig. 1B). Interestingly, three aspartic acid
4 residues: Asp-222, Asp-223 and Asp224 present in the same beta-strand that is located on the
5 outer surface of the wild type TPO contribute to a highly negatively-charged region which is
6 also conserved across many species including mice, rat and dog implying that this region is
7 crucial for the normal activity of the protein (Fig. 1C).
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14 Most cases of CH associated with alterations in the *TPO* gene were caused by either
15 homozygous or compound heterozygous mutations. In the present study, three different
16 alterations in the *TPO* gene were identified in CHP38 other than the confirmed
17 polymorphism. In addition to the c.2268dup, a novel, heterozygous mutation, c.1186C>T
18 (p.Arg396Cys), was detected in exon 8 and is expected to cause a substitution of arginine to
19 cysteine at codon 396 (Fig 2A). Results from both SIFT and Polyphen-2 analyses indicated
20 that the substitution is damaging and hence implying that this residue is important in the
21 structure/function of the TPO. A study has shown that the Arg-396 is one of the important
22 amino acids which could be involved in stabilizing the transition state of TPO protein during
23 the catalytic intermediate formation [14]. The formation of a stable catalytic intermediate
24 (compound I) of the TPO with H₂O₂ is crucial for thyroid hormone synthesis [15-16].
25 Catalytic process is initiated by the diffusion of H₂O₂ into the active site of the TPO protein.
26 The α -nucleophile H₂O₂ donates a proton to the distal imidazole ring (His-239) to form a
27 bond with the iron ion bound to residue His-494. After binding takes place, the protein attains
28 transition state to form compound I. The arginine at position 396 is believed to play a role in
29 stabilizing the charge for transition state of the protein through electrostatic interaction
30 [14,17]. Alternatively, it is believed that the arginine contributes to the abnormally low pKa
31 value of the distal histidine in the native resting enzyme. The changes of the pKa value in the
32 transition state of the distal imidaloze are the key to determine the effectiveness of the
33 catalysis process/rate of the compound I formation [17]. Therefore, a substitution from
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3 arginine to cysteine can bring devastating effects to the protein stability. In the present study,
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5 the 3-D model analysis showed that the p.Arg396Cys mutation has led to the structure
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7 alteration through the modification of the hydrogen bond network in the hydrophobic pocket
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9 which might interfere with the heme binding at Glu-399 [18] (Fig. 2B).
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12 Apart from the c.2268dup and c.1186C>T mutations, a non-synonymous substitution,
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14 c.2647C>T, was also identified in exon 16 of CHP38. The nucleotide alteration leads to a
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16 substitution of proline to serine at codon 883 in the C-terminal tail (Val-869 to Leu-933) of
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18 the TPO protein and was reported in patient with dysmorphogenetic CH in populations of
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20 Korea [19] and Japan [20] before. However, the consequence of the c.2647C>T remains
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22 ambiguous since both SIFT and Polyphen-2 analyses result [3] indicates that this alteration is
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24 “benign” suggesting that the Pro-883 is probably not critical for structure/function of the TPO
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26 and could be a rare polymorphism instead of disease-causing allele.
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33 It is worth noting that CHP18 and the two sisters reported in our previous study [6]
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35 with homozygous c.2268dup had developed large multinodular goiters in their mid or late
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37 adolescent years. Although it is not known whether the reduction in TPO expression due to
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39 c.2268dup can lead to increased risk of malignant transformation, other studies have shown
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41 cases of thyroid carcinoma has developed from congenital goiters that are associated with
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43 *TPO* mutation [21-22] or with lower/absence of TPO expression [23-24]. Therefore, it is
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45 important to have a careful surveillance for potential thyroid neoplasm in patients with
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47 c.2268dup mutation.
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52 CONCLUSION

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

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

Funding:

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Competing interests: None declared

Ethics approval: The University of Malaya Medical Centre (UMMC) Ethical Committee

Data sharing statement: No additional data are available

FIGURE-LEGEND

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 3-D orientation of the wild type (i) and mutant p.Asp224del (ii) 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 region. Residues Arg-223 to Tyr-226, Arg-648 and His-494 (iron binding site) are represented as Connolly surface to allow the visualization of the conformational changes in the TPO protein and its binding pocket. The Connolly surface is colored 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 TPO protein (ii). (C) Multiple-sequence alignment of human TPO with those of mouse, rat, pig, dog and chicken. The alignment data show that the negatively charge region (Asp-222, Asp-223 and Asp224) is conserved amongst human and many different animal species. The position of the deleted residue (p.Asp224del) is indicated by the arrow.

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 a

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3 substitution of arginine to cysteine at codon 396 (p.Arg396Cys). (B) Homology models
4 illustrating the 3-D orientation of the wild type (i) and mutant p.Arg396Cys (ii) TPO proteins.
5 The protein backbones are presented as ribbons (alpha-helix in red, beta-pleated sheet in cyan,
6 coils in grey, and turns in green). Hydrogen bonds are highlighted in: 1) green, hydrogen bond
7 under the normal criteria, 2) brown, hydrogen bond/salt bridge which forms between the O
8 atom of the carboxylate group and the H atom of an ammonium group in highly charged
9 region, 3) white, hydrogen bond between O atom of the carboxylate group and H atom on an
10 electro-positive C atom. Residues Ala-242, Arg-396/Cys-396, Ser398, Glu-399 (heme binding
11 site) and His-494 (iron binding site) are represented as Connolly surface to allow the
12 visualization of the conformational changes in the TPO protein and its binding pocket. The
13 Connolly surface is colored according to electrostatic potential spectrum (negative potential,
14 in red, to neutral, in white, to positive, in blue). Regions in yellow rings highlight the
15 interrupted hydrogen bond network observed when the wild type (i) changes to the mutant
16 TPO protein (ii).
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38 **Table 1:** Profiles of Malaysian-Chinese patients with CH showing the respective thyroid
39 function at the time of diagnosis. Gray shading indicates patients with the c.2268dup
40 mutation. Reference range for: TSH, cord = < 25.0 μ IU/ml; 1 to 3 days = 2.5 - 13.0 μ IU/ml;
41 less than a month = 0.6 - 10.0 μ IU/ml; 1 month onwards = 0.6 - 8.0 μ IU/ml, fT₄, cord = 28.4 -
42 68.4 pmol/L; 1 month = 20.0 - 28.4 pmol/L; 4 months onwards = 9.0 - 24.5 pmol/L and hTG,
43 (0-55.0 ng/ml). N/A: hTG level was not measured in patients below 12 years of age, unless
44 goiter is present. CHP51 and CHP55 were transferred to adult care and other hospital
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Table 1: Profiles of Malaysian-Chinese patients with CH showing the respective thyroid function at the time of diagnosis. Gray shading indicates patients with the c.2268dup mutation. Reference range for: TSH, cord = < 25.0 μ IU/ml; 1 to 3 days = 2.5 - 13.0 μ IU/ml; less than a month = 0.6 - 10.0 μ IU/ml; 1 month onwards = 0.6 - 8.0 μ IU/ml, fT₄, cord = 28.4 - 68.4 pmol/L; 1 month = 20.0 - 28.4 pmol/L; 4 months onwards = 9.0 - 24.5 pmol/L and hTG, (0-55.0 ng/ml). N/A: hTG level was not measured in patients younger than 12 years of age, unless goiter was present. CHP51 and CHP55 were transferred to adult care and other hospital respectively.

CH patient (CHP)	Gender	Age of diagnosis	Duration of follow-up (years)	TSH (μ IU/ml)	fT ₄ (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 goiter 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

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59	Male	3 months	15	>100	0.5	283	Normal
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Research

The prevalence of c.2268dup and detection identification of two novel alterations mutations, c.670_672del and c.1186C>T, and the prevalence of c.2268dup in the TPO gene in a cohort of Malaysian-Chinese with thyroid dysmorphogenesis

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ABSTRACT

Objectives: The c.2268dup mutation in the thyroid peroxidase (*TPO*) gene is the most common *TPO* ~~defect~~alteration reported in Taiwanese patients with thyroid dysmorphogenesis. 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 ~~*TPO* gene~~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 TSH and low fT₄ levels at the time of diagnosis with proven presence of thyroid gland

Primary outcome measure: Screening of the c.2268dup mutation in the *TPO* gene in all patients was carried out using 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 is a carrier for the c.2268dup mutation

Results: The c.2268dup mutation was detected in 4 out of the 12 patients. ~~A homozygous patient, CHP59 had developed large multinodular goiter at the age of 13.5 years which supports our previous findings that c.2268dup homozygotes developed dysmorphogenetic goiter in their mid or late adolescent years. Apart from the c.2268dup and a~~apart from the c.2268dup and a previously documented mutation, c.2647C>T, two novel *TPO* alterations mutations namely c.670_672del and c.1186C>T were also detected in our patients. *In silico* analyses predicted that the novel alterations mutations affect the structure/function of the TPO protein.

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7 **Conclusion:** The c.2268dup was detected in approximately one third of the Malaysian-
8 Chinese with thyroid dysmorphogenesis. The detection of the novel c.670_672del and
9 c.1186C>T alterations expand the mutation spectrum of *TPO* associated with thyroid
10 dysmorphogenesis.
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14 15 16 **Strengths and limitations of this study**

- 17
18 • The c.2268dup was detected in approximately one third of the Malaysian-Chinese
19 with thyroid dysmorphogenesis. The c.2268dup in the homozygous form might be
20 associated with the phenotype of dysmorphogenetic goiter.
21
- 22 • Two novel *TPO* mutations alterations namely c.670_672del and c.1186C>T were also
23 detected in this study. *In silico* analyses revealed that the two mutations alterations
24 may affect the normal structure/function of the mutant TPO protein.
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- 26 • The *in silico* functional analyses could not be further validated due to unavailability of
27 thyroid tissue samples from the patients.
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51 **Keywords:** Congenital hypothyroidism, *thyroid peroxidase*, c.2268dup, novel mutations,
52 Malaysian-Chinese
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INTRODUCTION

Congenital hypothyroidism (CH) is one of the most common endocrine disorders in the world affecting 1 in 3000 - 4000 newborn babies ~~wherewith~~ 10 - 20 % of the cases ~~wereare~~ due to thyroid dysmorphogenesis [1]. Over the past three decades, numerous cases of dysmorphogenetic CH have been linked to ~~defeetsalterations~~ in the *TPO* gene [2-3]. This gene encodes a protein with 933 amino acids in length which plays an important role in thyroid hormones synthesis [4]. Niu *et al.* in 2002 reported a nonsense mutation, c.2268dup, a common cause of dysmorphogenetic CH in Taiwan with molecular proof of a founder effect ~~where the ancestors of these patients originated from the southern province of China [4]. [5].~~ Recently, we identified the c.2268dup mutation in a Malaysian-Chinese family with goitrous CH and showed that the mutation leads to the reduction of TPO protein expression with a consequential loss of enzyme activity [6]. The Chinese forms the second largest ethnic group which constitutes about 24.6 % of the 28.3 million Malaysian populations [7]. As the cause of dysmorphogenetic CH in Malaysian-Chinese remains unclear, we therefore embarked on this present study with the aim of providing further data on the incidence of ~~*TPO* gene~~ ~~c.2268dup mutation~~ in Malaysian Chinese and ~~its~~ possible phenotypic effects.

SUBJECTS AND METHODS

~~Subjects for the *TPO* mutation screening~~

A cohort (duration of follow-up between 3 and 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. ~~Mutational screening was performed on genomic DNA extracted from peripheral venous blood of all patients who~~ ~~None of the patients are from a consanguineous family. All patients~~ had high TSH and low fT₄ levels at the time of diagnosis with proven presence of thyroid gland (Table

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7 1). Serum thyroglobulin (hTG) level was measured in patients who have reached puberty (12
8 years old and older) or presented with goiter; except for CHP51 and CHP55 who had been
9 transferred to adult care and other hospital, respectively (Table 1). Informed written consent
10 was obtained either from patients or their parent/guardian. This study was approved by the
11 UMMC Ethical Committee (Institutional Review Board) in accordance to the ICH-GCP
12 guideline and the Declaration of Helsinki (Reference number, 654.16). Perchlorate discharge
13 test was however, not performed in our patients since permission was not granted by the
14 majority of the patients' parents.

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23 TPO mutation screening†).

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25 PCR amplification and direct DNA sequencing were performed to screen for alteration
26 of *TPO* gene using genomic DNA extracted from peripheral venous blood. A forward (5'-
27 ACAGGGACGTTGGTGTGTGG-3') and a reverse (5'-TCAGAAGCACCTTTTGCG-3')
28 primer were used to PCR-amplify exon 13 of the *TPO* gene (NM_000547.5) where the
29 c.2268dup mutation is located. Further screening for mutations in other exonic regions of the
30 *TPO* gene [8] was carried out if the patient is a carrier for the c.2268dup mutation. To confirm
31 that an alteration in the *TPO* gene is due to a disease-causing mutation instead of a
32 polymorphism, a total of 100 chromosomes from 50 unrelated healthy individuals were also
33 screened for the same mutation. Informed written consent was obtained either from patients or
34 their parent/guardian. This study was approved by the UMMC Ethical Committee
35 (Institutional Review Board) in accordance to the ICH-GCP guideline and the Declaration of
36 Helsinki (Reference number, 654.16).

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48 *In silico* analyses of the novel c.670_672del (p.Asp224del) and c.1186C>T (p.Arg396Cys)
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The effects of the novel mutations on normal TPO activity were evaluated using SIFT and Polyphen-2 algorithms. Alignment of 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). The homology models of human TPO including the wild type and the two mutant proteins: p.Asp224del and p.Arg396Cys were generated, verified and compared as described before [8].

RESULTS AND DISCUSSION

~~The~~ In addition to a patient with homozygous c.2268dup mutation was detected in 4 out of the total 12 unrelated patients in this study. Two patients, CHP18 and CHP59, were homozygotes while another two, CHP38 and CHP58, were heterozygotes for the mutation. In addition to a case reported in our previous study [6], the c.2268dup was detected in only 31 % of the total alleles studied, lower than what were reported in the studies by Niu *et al.* (2002) (86 %) [4] and Wu *et al.* (2002) (40 %) [8]. For their study, only. So far, the mutation was only detected amongst patients with confirmed total iodide organification defect (TIOD) through tested using perchlorate discharge test were screened for the TPO gene mutations. In contrast, the perchlorate [5, 9]. The test for TIOD was however, not performed in our patients since permission was not granted by the majority of the patients' parents. This could be the reason for the higher prevalence of the c.2268dup mutation in the Taiwanese patients when compared to our study: [5, 9]. Nonetheless, the difference in the origin between the Chinese population of Malaysia [10] and Taiwan [11] may also have contributed to this variation.

Further studies on the c.2268dup mutation by increasing sample size and collecting information on the ancestral origins of the patients is expected to gain a deeper understanding of the frequency and distribution pattern of the c.2268dup mutation in Malaysian-Chinese

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7 **population.** Two patients in this study, CHP18 and CHP59, were homozygotes while another
8 two, CHP38 and CHP58, were heterozygotes for the mutation.

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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 3 nucleotides (GAC) is predicted to produce an in-frame deletion of a single aspartic acid (p.Asp224del) in the TPO protein (Fig. 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 brought conformational changes to the protein by shortening the length of the beta-strand and also disrupted the correct orientation of hydrogen bonds 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 (heme axial ligand) binding site [12], it could possibly interfere with binding of the iron ion at His-494 or the electron transfer activity of TPO wherein which His-494 is the source of the electron [13]. It is also predicted that the deletion of Asp-224 will alter the orientation of a salt bridge between Asp-223 and Arg-648 (Fig. 1B). Interestingly, three aspartic acid residues: Asp-222, Asp-223 and Asp224 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 (Fig. 1C).

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Most cases of CH associated with defects/alterations in the *TPO* gene were caused by either homozygous or compound heterozygous mutations. In the present study, three different mutations/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 a substitution of arginine to cysteine at codon 396 (Fig 2A). Results from both SIFT and Polyphen-2 analyses indicated

that the substitution is damaging and hence implying that this residue is important in the structure/function of the TPO. A study has shown that the Arg-396 is one of the important amino acids which could be involved in stabilizing the transition state of TPO protein during the catalytic intermediate formation [14]. The formation of a stable catalytic intermediate (compound I) of the TPO with H₂O₂ is crucial for thyroid hormone synthesis [15-16]. Catalytic process is initiated by the diffusion of H₂O₂ into the active site of the TPO protein. The α -nucleophile H₂O₂ 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 takes place, the protein attains transition state to form compound I. The arginine at position 396 is believed to play a role in stabilizing the charge for transition state of the protein through electrostatic interaction [14,17]. 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 of the pKa value in the transition state of the distal imidazole are the key to determine the effectiveness of the catalysis process/rate of the compound I formation [17]. Therefore, a substitution from arginine to cysteine can bring devastating effects to the protein stability. In the present study, the 3-D model analysis showed that the p.Arg396Cys mutation has led to the structure alteration through the modification of the hydrogen bond network in the hydrophobic pocket which might interfere with the heme binding at Glu-399 [18] (Fig. 2B).

~~— Apart from the c.2268dup and c.1186C>T mutations, a non-synonymous substitution, c.2647C>T mutation, was also identified in exon 16 of CHP38, where it. The nucleotide alteration leads to a substitution of proline to serine at codon 883 in the C-terminal tail (Val-869 to Leu-933) of the TPO protein, and was reported in patient with dyshormonogenetic CH in populations of Korea [19] and Japan [20] before. However, the consequence of the c.2647C>T remains ambiguous since both SIFT and Polyphen-2 analyses result from a previous study [31] indicates that this mutational alteration is “benign” suggesting~~

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7 that the Pro-883 is probably not critical for structure/function of the TPO and does not modify
8 TPO function. Interestingly, this mutation had also been reported in populations of Korea [18]
9 and Japan [19]. Whether the c.2647C>T mutation is possibly a rare polymorphism is not
10 known. Despite the *in silico* findings indicated that the substitution of Pro-883 to serine might
11 not be critical to the protein structure/function, previously reported cases showed that patients
12 associated with this mutation presented with severe dysmorphogenetic CH [18-19]. Since
13 Human Splicing Finder (HSF) analysis predicted that the c.2647C>T mutation interrupts the
14 sequences recognized by ESE proteins (data not shown), we therefore could not exclude that
15 the sequence alteration might possibly regulate the splicing activity of the TPO pre-mRNA
16 and lead to CH in these patients. could be a rare polymorphism instead of disease-causing
17 allele.

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30 It is worth noting that patients with homozygous c.2268dup inclusive of CHP18 and
31 the two patients/sisters reported in our previous study [6], except for patient CHP59 who was
32 12-year-old at the time of the study,] with homozygous c.2268dup had developed large
33 multinodular goiter/goiters in their mid or late adolescent years. Although it is not known
34 whether the reduction in TPO expression due to c.2268dup can lead to increased risk of
35 malignant transformation, other studies have shown cases of thyroid carcinoma has developed
36 from congenital goiter/goiters that is/are associated with *TPO* mutation [21-22] or with lower/
37 absence of TPO expression [23-24]. Therefore, it is important to have a careful surveillance
38 for potential thyroid neoplasm in patients with c.2268dup mutation.

49 CONCLUSION

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

For peer review only

Contributors:

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

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Competing interests: None declared

Ethics approval: The University of Malaya Medical Centre (UMMC) Ethical Committee

Data sharing statement: No additional data are available

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FIGURE-LEGEND

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 3-D orientation of the wild type (i) and mutant p.Asp224del (ii) 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 region. Residues Arg-223 to Tyr-226, Arg-648 and His-494 (iron binding site) are represented as Connolly surface to allow the visualization of the conformational changes in the TPO protein and its binding pocket. The Connolly surface is colored 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 TPO protein (ii). (C) Multiple-sequence alignment of human TPO with those of mouse, rat, pig, dog and chicken. The alignment data show that the negatively charge region (Asp-222, Asp-223 and Asp224) is conserved amongst human and many different animal species. The position of the deleted residue (p.Asp224del) is indicated by the arrow.

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 a substitution of arginine to cysteine at codon 396 (p.Arg396Cys). (B) Homology models

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16 region, 3) white, hydrogen bond between O atom of the carboxylate group and H atom on an
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18 electro-positive C atom. Residues Ala-242, Arg-396/Cys-396, Ser398, Glu-399 (heme binding
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20 site) and His-494 (iron binding site) are represented as Connolly surface to allow the
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35 **Table 1: Profiles of Malaysian-Chinese patients with CH showing the respective thyroid**
36 **function at the time of diagnosis. Gray shading indicates patients with the c.2268dup**
37 **mutation. Reference range for: TSH, cord = < 25.0 µIU/ml; 1 to 3 days = 2.5 - 13.0 µIU/ml;**
38 **less than a month = 0.6 - 10.0 µIU/ml; 1 month onwards = 0.6 - 8.0 µIU/ml, fT₄, cord = 28.4 -**
39 **68.4 pmol/L; 1 month = 20.0 - 28.4 pmol/L; 4 months onwards = 9.0 - 24.5 pmol/L and hTG,**
40 **(0-55.0 ng/ml). N/A: hTG level was not measured in patients below 12 years of age, unless**
41 **goiter is present. CHP51 and CHP55 were transferred to adult care and other hospital**
42 **respectively.**
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Table 1: Profiles of Malaysian-Chinese patients with CH showing the respective thyroid function at the time of diagnosis. Gray shading indicates patients with the c.2268dup mutation. Reference range for: TSH, cord = < 25.0 µIU/ml; 1 to 3 days = 2.5 - 13.0 µIU/ml; less than a month = 0.6 - 10.0 µIU/ml; 1 month onwards = 0.6 - 8.0 µIU/ml, fT₄, cord = 28.4 - 68.4 pmol/L; 1 month = 20.0 - 28.4 pmol/L; 4 months onwards = 9.0 - 24.5 pmol/L and hTG, (0-55.0 ng/ml). N/A: hTG level was not measured in patients younger than 12 years of age, unless goiter was present. CHP51 and CHP55 were transferred to adult care and other hospital respectively.

CH patient (CHP)	Gender	Age of diagnosis	Duration of follow-up (years)	TSH (µIU/ml)	fT ₄ (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 goiter 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

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59	Male	3 months	15	>100	0.5	283	Normal
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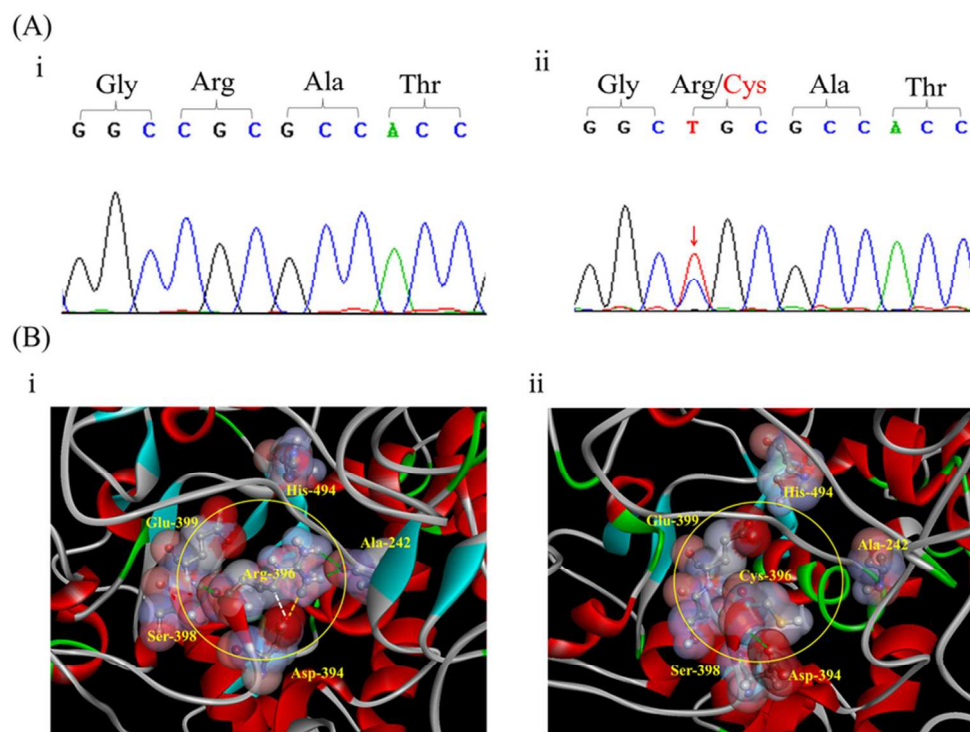


Figure 2

(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 a substitution of arginine to cysteine at codon 396 (p.Arg396Cys). (B) Homology models illustrating the 3-D orientation of the wild type (i) and mutant p.Arg396Cys (ii) 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 region, 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, Ser398, Glu-399 (heme binding site) and His-494 (iron binding site) are represented as Connolly surface to allow the visualization of the conformational changes in the TPO protein and its binding pocket. The Connolly surface is colored 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 TPO protein (ii).

78x60mm (300 x 300 DPI)