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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 dyshormonogenesis. 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 dyshormonogenetic 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 dyshormonogenesis. The detection of the novel c.670_672del and

c.1186C>T alterations expand the mutation spectrum of *TPO* associated with thyroid dyshormonogenesis.

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* mutations namely c.670_672del and c.1186C>T were also detected in this study. *In silico* analyses revealed that the two mutations 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 where 10 - 20 % of the cases were due to thyroid dyshormonogenesis [1]. Over the past three decades, numerous cases of dyshormonogenetic CH have been linked to defects in the *TPO* gene [2]. This gene encodes a protein with 933 amino acids in length which plays an important role in thyroid hormones synthesis [3]. 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 where the ancestors of these patients originated from the southern province of China [4]. 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 [5]. The Chinese forms the second largest ethnic group which constitutes about 24.6 % of the 28.3 million Malaysian populations [6]. 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 *TPO* gene 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 unrelated Chinese patients with dyshormonogenetic 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 had high TSH and low fT_4 levels at the time of diagnosis with proven presence of thyroid gland (Table S1). A forward (5'-ACAGGGACGTTGGTGTGTGG-3') and a

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

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

The effects of the novel mutations on normal TPO activity were evaluated using SIFT 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 [7].

RESULTS AND DISCUSSION

The 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 [5] , 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 patients with confirmed total iodide organification defect (TIOD) through perchlorate discharge test were screened for the *TPO* gene mutations. In contrast, the perchlorate test was 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. Nonetheless, the difference in the origin between the Chinese population of Malaysia [9] and Taiwan [10] may also have contributed to this variation.

Apart from the c.2268dup mutation, a novel 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 [11], it could possibly interfere with binding of the iron ion at His-494 or the electron transfer activity of TPO where His-494 is the source of the electron [12]. 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).

Most cases of CH associated with defects in the *TPO* gene were caused by either homozygous or compound heterozygous mutations. In the present study, three different mutations in *TPO* gene were identified in CHP38. In addition to the c.2268dup, a novel 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 [13]. The formation of a stable catalytic intermediate (compound I) of the TPO with H_2O_2 is crucial for thyroid hormone synthesis [14-15]. Catalytic process is initiated by the diffusion of H_2O_2 into the active site of the TPO protein. The α-nucleophile H_2O_2 donates a proton to the distal imidazole ring (His-239) to form a bond with the iron ion bound to residue His-494. After binding 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 [13,16]. 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 imidaloze are the key to determine the effectiveness of the catalysis process/rate of the compound I formation [16]. 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 [17] (Fig. 2B).

Apart from the c.2268dup and c.1186C>T mutations, a c.2647C>T mutation was also identified in exon 16 of CHP38, where it 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. SIFT and Polyphen-2 analyses result from a previous study [2] indicates that this mutation is "benign" suggesting that the Pro-883 is probably not critical for structure/function of the TPO and does not modify TPO function. Interestingly, this mutation had also been reported in populations of Korea [18] and Japan [19]. Whether the c.2647C>T mutation is possibly a rare polymorphism is not known. Despite the *in silico* findings indicated that the substitution of Pro-883 to serine might not be critical to the protein structure/function, previously reported cases showed that patients associated with this mutation presented with severe dyshormonogenetic CH [18-19]. Since Human Splicing Finder (HSF) analysis predicted that the c.2647C>T mutation interrupts the sequences recognized by ESE proteins (data not shown), we therefore could not exclude that the sequence alteration might possibly regulate the splicing activity of the TPO pre-mRNA and lead to CH in these patients.

It is worth noting that patients with homozygous c.2268dup inclusive of two patients reported in our previous study [5], except for patient CHP59 who was 12-year old at the time of the study, had developed large multinodular goiter in their mid or late adolescent years. Although it is not known whether the reduction in TPO expression due to c.2268dup can lead to increased risk of malignant transformation, other studies have shown cases of thyroid carcinoma has developed from congenital goiter that is associated with *TPO* mutation [20-21] or lower/ absence of TPO expression [22-23]. Therefore, it is important to have a careful surveillance for potential thyroid neoplasm in patients with c.2268dup mutation.

CONCLUSION

In conclusion, we report two novel mutations in the *TPO* gene, c.670_672del and c.1186C>T, and reveal the association of c.2268dup mutation with approximately one third

of a cohort of Malaysian-Chinese with dyshormonogenetic CH. This study also support our previous findings that c.2268dup homozygotes developed dyshormonogenetic goiter in their mid or late adolescent years. These data will be useful in diagnosing or predicting goitrous dyshormonogenetic CH.

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

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 it's 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

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).

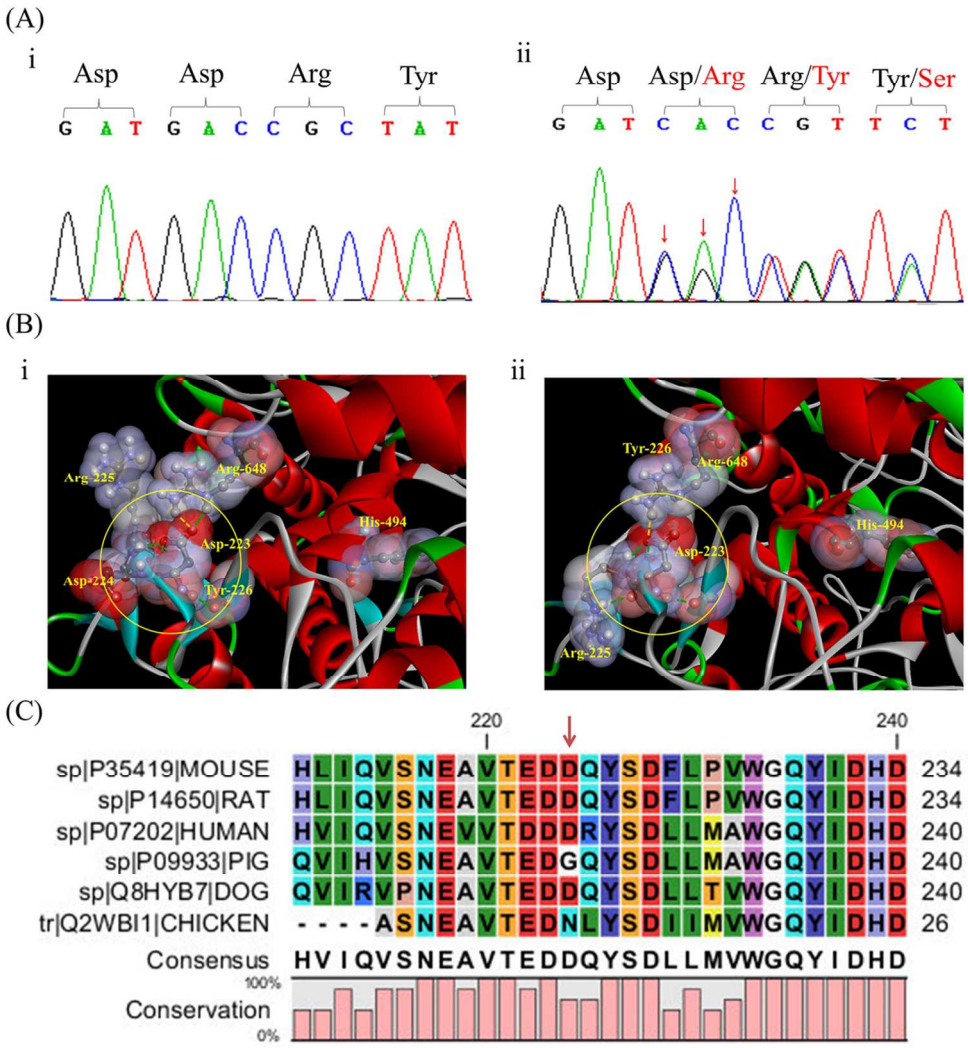

Figure 1: (A) DNA sequencing profiles. Electropherogram profiles of a control with a wild type allele (i), and CHP51 who is a heterozygote for the c.670_672del mutation (ii). The three deleted nucleotides (GAC) are indicated by the arrows. The sequence alteration is predicted to produce an in-frame deletion 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 it's 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.
110x119mm (300 x 300 DPI)

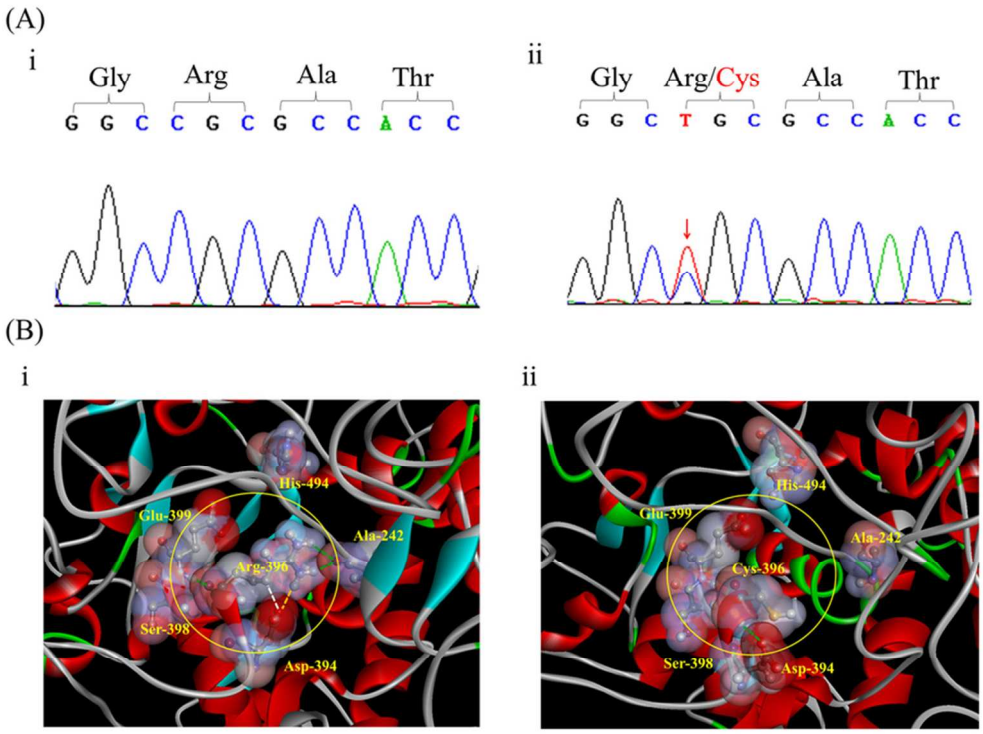

Figure 2

(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 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 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 (µIU/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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Research

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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 dyshormonogenesis. 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 dyshormonogenesis. The detection of the novel c.670_672del and c.1186C>T alterations expand the mutation spectrum of *TPO* associated with thyroid dyshormonogenesis.

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* mutations namely c.670_672del and c.1186C>T were also detected in this study. *In silico* analyses revealed that the two mutations 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]. This gene encodes a protein with 933 amino acids in length which plays an important role in thyroid hormones synthesis [3]. 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 [4]. 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 [5]. The Chinese forms the second largest ethnic group which constitutes about 24.6 % of the 28.3 million Malaysian populations [6]. 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 fT_4 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 hospital, respectively (Table 1). Informed written consent was obtained either from patients or their parent/guardian. This study was approved by the UMMC Ethical Committee (Institutional Review Board) in accordance to the ICH-GCP guideline and the Declaration of Helsinki (Reference number, 654.16). Perchlorate discharge test was however, not performed in our patients since permission was not granted by the majority of the patients' parents.

TPO mutation screening

PCR amplification and direct DNA sequencing were performed to screen for alteration of *TPO* gene using genomic DNA extracted from peripheral venous blood. A forward (5'-ACAGGGACGTTGGTGTGTGG-3') and a reverse (5'-TCAGAAGCACCTTTTGGCG-3') primer were used to PCR-amplify exon 13 of the *TPO* gene (NM_000547.5) where the c.2268dup mutation is located. Further screening for mutations in other exonic regions of the *TPO* gene [7] was carried out if the patient is a carrier for the c.2268dup mutation. To confirm that an alteration in the *TPO* gene is due to a disease-causing mutation instead of a polymorphism, a total of 100 chromosomes from 50 unrelated healthy individuals were also screened for the same mutation.

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

The effects of the novel mutations on normal TPO activity were evaluated using SIFT 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 [7].

RESULTS AND DISCUSSION

In addition to a patient with homozygous c.2268dup mutation reported in our previous study [5], 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 [4, 8]. 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 [4, 8]. Nonetheless, the difference in the origin between the Chinese population of Malaysia [9] and Taiwan [10] may also have contributed to this variation. 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 [11], 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 [12]. 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).

Most cases of CH associated with alterations in the *TPO* gene were caused by either homozygous or compound heterozygous mutations. In the present study, three different alterations in the *TPO* gene were identified in CHP38 other than the confirmed polymorphism. In addition to the c.2268dup, a novel, heterozygous mutation, c.1186C>T (p.Arg396Cys), was detected in exon 8 and is expected to cause 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 [13]. The formation of a stable catalytic intermediate (compound I) of the TPO with H_2O_2 is crucial for thyroid hormone synthesis [14-15]. Catalytic process is initiated by the diffusion of H_2O_2 into the active site of the TPO protein. The α-nucleophile H_2O_2 donates a proton to the distal imidazole ring (His-239) to form a bond with the iron ion bound to residue His-494. After binding 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 [13,16]. 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 imidaloze are the key to determine the effectiveness of the catalysis process/rate of the compound I formation [16]. 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 [17] (Fig. 2B).

Apart from the c.2268dup and c.1186C>T mutations, a non-synonymous substitution, c.2647C>T, was also identified in exon 16 of CHP38. The nucleotide alteration leads to 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 [18] and Japan [19] before. However, the consequence of the c.2647C>T remains ambiguous since both SIFT and Polyphen-2 analyses result [2] indicates that this alteration is "benign" suggesting that the Pro-883 is probably not critical for structure/function of the TPO and could be a rare polymorphism instead of disease-causing allele.

It is worth noting that CHP18 and the two sisters reported in our previous study [5] with homozygous c.2268dup had developed large multinodular goiters in their mid or late adolescent years. Although it is not known whether the reduction in TPO expression due to c.2268dup can lead to increased risk of malignant transformation, other studies have shown cases of thyroid carcinoma has developed from congenital goiters that are associated with *TPO* mutation [20-21] or with lower/absence of TPO expression [22-23]. Therefore, it is important to have a careful surveillance for potential thyroid neoplasm in patients with c.2268dup mutation.

CONCLUSION

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

mid or late adolescent years. These data will be useful in diagnosing or predicting goitrous dyshormonogenetic CH.

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

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 it's 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

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).

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, 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 and hTG, (0-55.0 ng/ml). N/A: hTG level was not measured in patients below 12 years of age, unless goiter is present. CHP51 and CHP55 were transferred to adult care and other hospital respectively.

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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

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 dyshormonogenesis. 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.

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

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* mutations namely c.670_672del and c.1186C>T were also detected in this study. *In silico* analyses revealed that the two mutations may affect the normal structure/function of the mutant TPO protein.
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INTRODUCTION

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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 dyshormonogenetic 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 fT_4 levels at the time of diagnosis with proven presence of thyroid gland (Table

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

TPO mutation screening~~S1).~~

PCR amplification and direct DNA sequencing were performed to screen for alteration of *TPO* gene using genomic DNA extracted from peripheral venous blood. A forward (5'-ACAGGGACGTTGGTGTGTGG-3') and a reverse (5'-TCAGAAGCACCTTTTGGCG-3') primer were used to PCR-amplify exon 13 of the *TPO* gene (NM_000547.5) where the c.2268dup mutation is located. Further screening for mutations in other exonic regions of the *TPO* gene [7] was carried out if the patient is a carrier for the c.2268dup mutation. To confirm that an alteration in the *TPO* gene is due to a disease-causing mutation instead of a polymorphism, a total of 100 chromosomes from 50 unrelated healthy individuals were also screened for the same mutation. ~~Informed written consent was obtained either from patients or their parent/guardian. This study was approved by the UMMC Ethical Committee (Institutional Review Board) in accordance to the ICH-GCP guideline and the Declaration of Helsinki (Reference number, 654.16).~~

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

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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 [7].

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 [5~~],~~], 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~~ [4, 8]. 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~~.~~ [4, 8]. Nonetheless, the difference in the origin between the Chinese population of Malaysia [9] and Taiwan [10] may also have contributed to this variation. Two patients in this study, CHP18 and CHP59, were homozygotes while another 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 [11], it could possibly interfere with binding of the iron ion at His-494 or the electron transfer activity of TPO ~~where~~in which His-494 is the source of the electron [12]. 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).

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

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the catalytic intermediate formation [13]. The formation of a stable catalytic intermediate (compound I) of the TPO with H_2O_2 is crucial for thyroid hormone synthesis [14-15]. Catalytic process is initiated by the diffusion of H_2O_2 into the active site of the TPO protein. The α-nucleophile H_2O_2 donates a proton to the distal imidazole ring (His-239) to form a bond with the iron ion bound to residue His-494. After binding 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 [13,16]. 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 imidaloze are the key to determine the effectiveness of the catalysis process/rate of the compound I formation [16]. 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 [17] (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 [18] and Japan [19] before. However, the consequence of the c.2647C>T remains ambiguous since both SIFT and Polyphen-2 analyses result ~~from a previous study~~ [2] indicates that this ~~mutation~~alteration is "benign" suggesting that the Pro-883 is probably not critical for structure/function of the TPO and ~~does not modify TPO function. Interestingly, this mutation had also been reported in populations of Korea [18] and Japan [19]. Whether the c.2647C>T mutation is possibly a rare polymorphism~~

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

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

CONCLUSION

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

mid or late adolescent years. These data will be useful in diagnosing or predicting goitrous dyshormonogenetic CH.

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

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 it's 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

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).

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, 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 and hTG, (0-55.0 ng/ml). N/A: hTG level was not measured in patients below 12 years of age, unless goiter is present. CHP51 and CHP55 were transferred to adult care and other hospital respectively.

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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

59	Male	3 months	15	>100	0.5	283	Normal

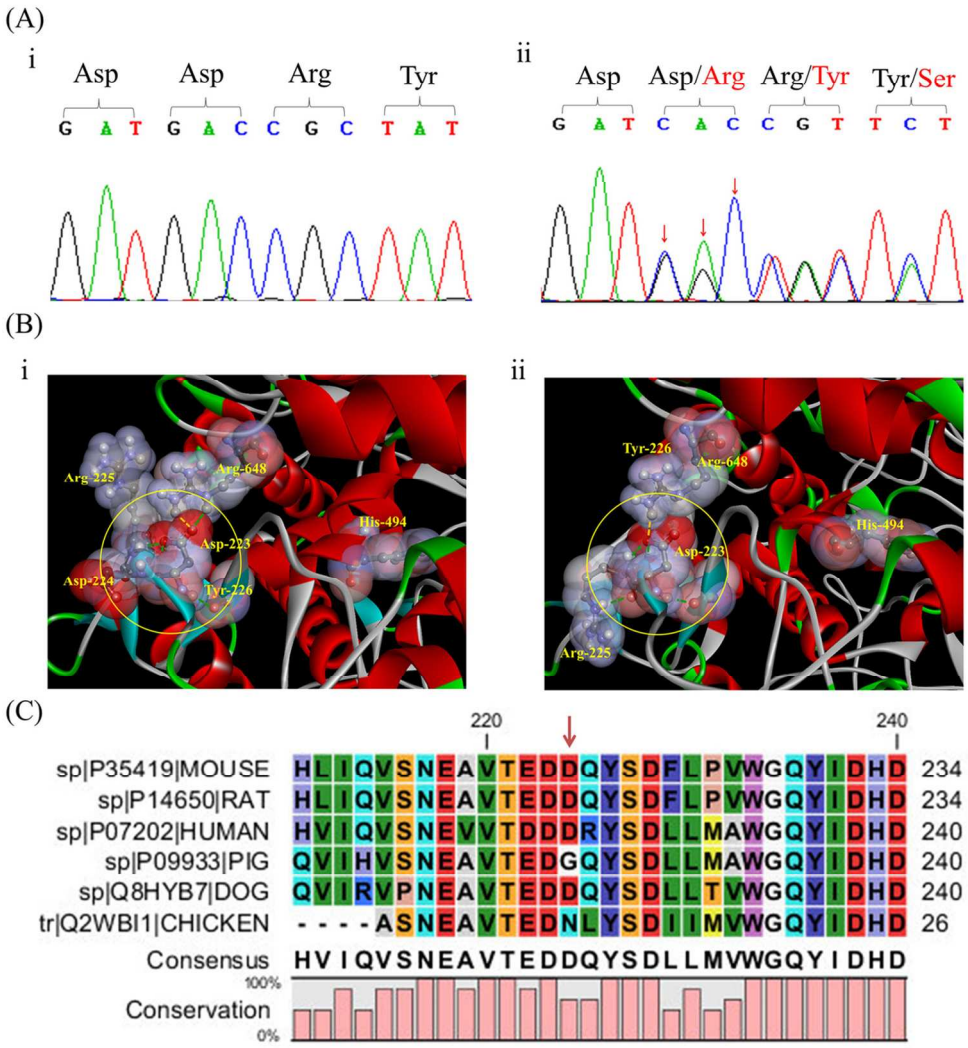

Figure 1: (A) DNA sequencing profiles. Electropherogram profiles of a control with a wild type allele (i), and CHP51 who is a heterozygote for the c.670_672del mutation (ii). The three deleted nucleotides (GAC) are indicated by the arrows. The sequence alteration is predicted to produce an in-frame deletion 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 it's 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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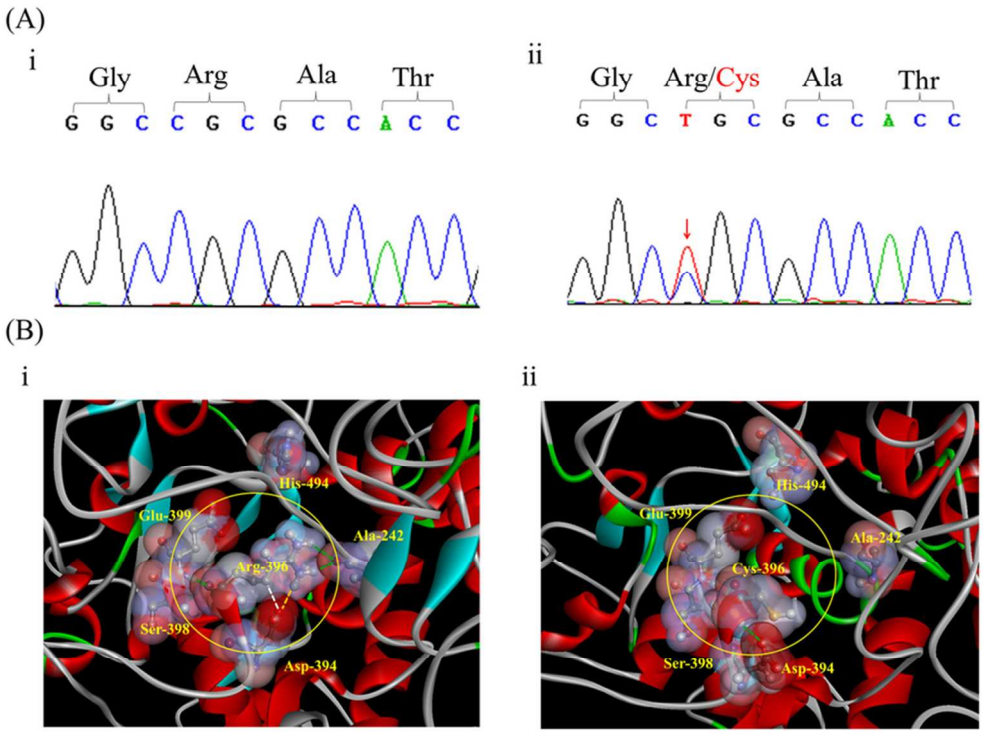

Figure 2
(A) DNA sequencing profiles. Electropherogram profiles of a control with a wild type allele (i), and CHP38 who is a heterozygote for the c.1186C>T mutation (ii). The single nucleotide transition is indicated by the arrow. The sequence alteration is predicted to cause a substitution of arginine 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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Research

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 dyshormonogenesis. 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* 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 dyshormonogenesis. The detection of the novel c.670_672del and c.1186C>T alterations expand the mutation spectrum of *TPO* associated with thyroid dyshormonogenesis.

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 fT_4 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

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

TPO mutation screening

PCR amplification and direct DNA sequencing were performed to screen for alteration of *TPO* gene using genomic DNA extracted from peripheral venous blood. A forward (5'-ACAGGGACGTTGGTGTGTGG-3') and a reverse (5'-TCAGAAGCACCTTTTGGCG-3') primer were used to PCR-amplify exon 13 of the *TPO* gene (NM_000547.5) where the c.2268dup mutation is located. Further screening for mutations in other exonic regions of the *TPO* gene [8] was carried out if the patient is a carrier for the c.2268dup mutation. To confirm that an alteration in the *TPO* gene is due to a disease-causing mutation instead of a polymorphism, a total of 100 chromosomes from 50 unrelated healthy individuals were also screened for the same mutation.

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

The effects of the novel mutations on normal TPO activity were evaluated using SIFT 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

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

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).

Most cases of CH associated with alterations in the *TPO* gene were caused by either homozygous or compound heterozygous mutations. In the present study, three different alterations in the *TPO* gene were identified in CHP38 other than the confirmed polymorphism. In addition to the c.2268dup, a novel, heterozygous mutation, c.1186C>T (p.Arg396Cys), was detected in exon 8 and is expected to cause 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 H_2O_2 is crucial for thyroid hormone synthesis [15-16]. Catalytic process is initiated by the diffusion of H_2O_2 into the active site of the TPO protein. The α-nucleophile H_2O_2 donates a proton to the distal imidazole ring (His-239) to form a bond with the iron ion bound to residue His-494. After binding 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 imidaloze 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, was also identified in exon 16 of CHP38. 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 [3] indicates that this alteration is "benign" suggesting that the Pro-883 is probably not critical for structure/function of the TPO and could be a rare polymorphism instead of disease-causing allele.

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

CONCLUSION

In conclusion, we report two novel alterations which are likely pathogenic in the *TPO* gene, c.670_672del and c.1186C>T, and an association of c.2268dup mutation with

approximately one third of a cohort of Malaysian-Chinese with dyshormonogenetic CH. This study also supports our previous findings that c.2268dup homozygotes developed dyshormonogenetic goiter in their mid or late adolescent years. These data will be useful in diagnosing or predicting goitrous dyshormonogenetic CH.

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 it's 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 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).

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, 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 and hTG, (0-55.0 ng/ml). N/A: hTG level was not measured in patients below 12 years of age, unless goiter is present. CHP51 and CHP55 were transferred to adult care and other hospital 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, 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 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

6	59	Male	3 months	15	>100	0.5	283	Normal

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 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 dyshormonogenesis. 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 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. ~~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 dyshormonogenetic 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 ~~mutations~~ alterations affect the structure/function of the TPO protein.

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

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* ~~mutations~~ alterations namely c.670_672del and c.1186C>T were also detected in this study. *In silico* analyses revealed that the two ~~mutations~~ alterations may affect the normal structure/function of the mutant TPO protein.
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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 dyshormonogenetic 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 fT_4 levels at the time of diagnosis with proven presence of thyroid gland (Table

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TPO mutation screening~~S1).~~

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

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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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population. Two patients in this study, CHP18 and CHP59, were homozygotes while another 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 ~~where~~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 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 H_2O_2 is crucial for thyroid hormone synthesis [15-16]. Catalytic process is initiated by the diffusion of H_2O_2 into the active site of the TPO protein. The α-nucleophile H_2O_2 donates a proton to the distal imidazole ring (His-239) to form a bond with the iron ion bound to residue His-494. After binding 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 imidaloze 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~~ [3] indicates that this ~~mutation~~alteration is “benign” suggesting

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

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

CONCLUSION

In conclusion, we report two novel alterations which are likely pathogenic in the *TPO* gene, c.670_672del and c.1186C>T, and reveal the association of c.2268dup mutation with

approximately one third of a cohort of Malaysian-Chinese with dyshormonogenetic CH. This study also support our previous findings that c.2268dup homozygotes developed dyshormonogenetic goiter in their mid or late adolescent years. These data will be useful in diagnosing or predicting goitrous dyshormonogenetic CH.

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

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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 it's 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

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).

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, 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 and hTG, (0-55.0 ng/ml). N/A: hTG level was not measured in patients below 12 years of age, unless goiter is present. CHP51 and CHP55 were transferred to adult care and other hospital respectively.

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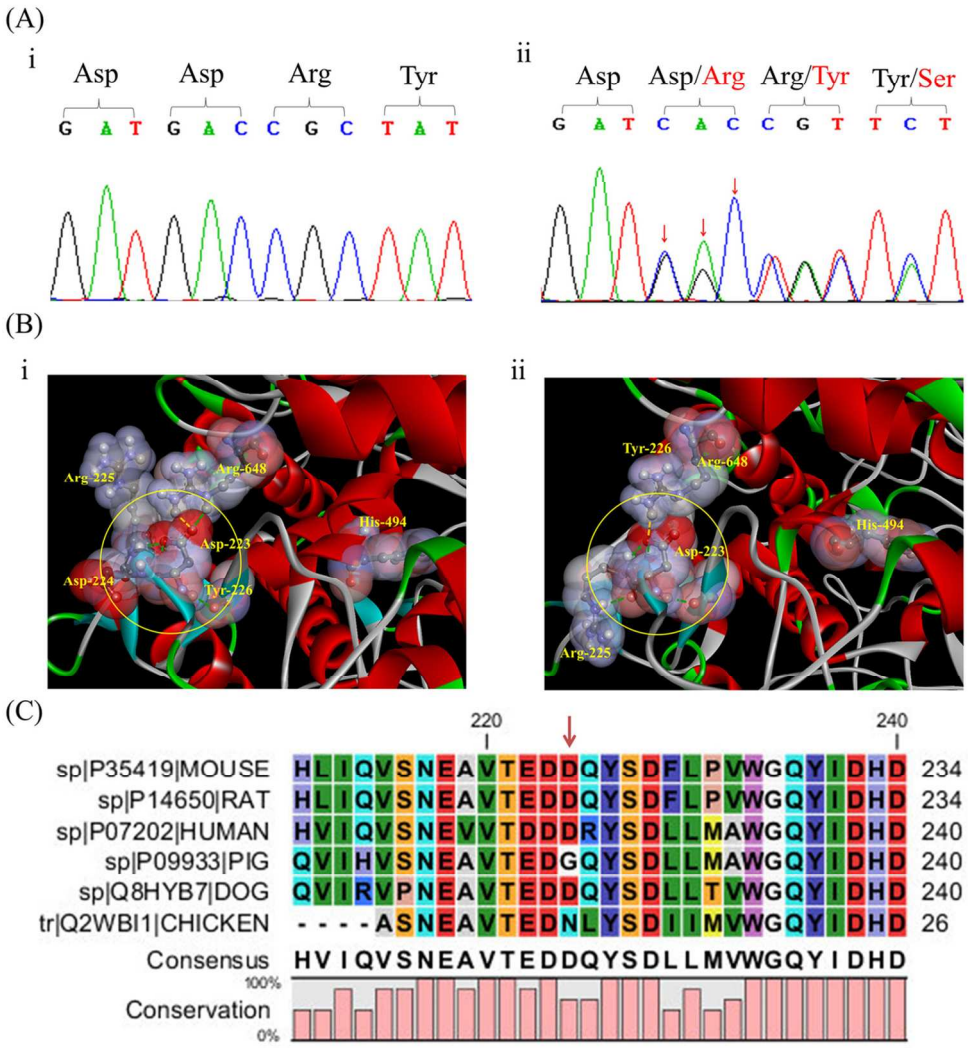
CH patient (CHP)	Gender	Age of diagnosis	Duration of follow-up (years)	TSH (µIU/ml)	fT_4 (pmol/L)	hTG (ng/ml)	Thyroid scan (at 3 years old) / ultrasonography
16	Female	10 days	17	26.5	11.3	40.7	Normal
18	Male	3 weeks	20	59.0	13.0	26	Multinodular goiter at 13.5 years
21	Female	3 months	13	20.6	14.1	20	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

59	Male	3 months	15	>100	0.5	283	Normal



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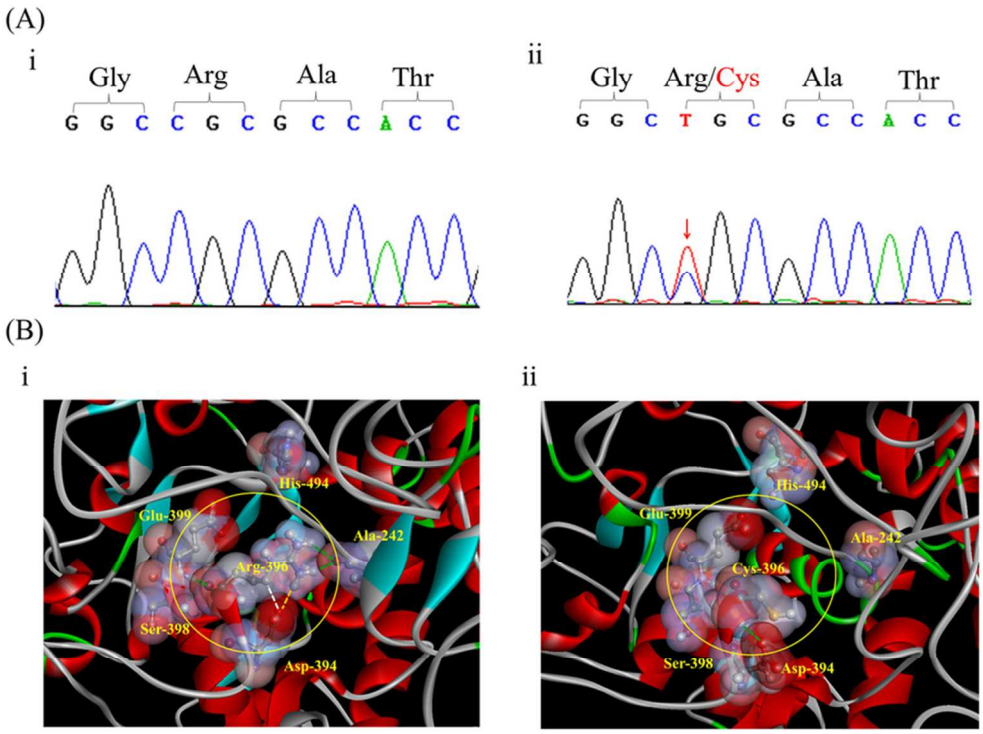

Figure 2
(A) DNA sequencing profiles. Electropherogram profiles of a control with a wild type allele (i), and CHP38 who is a heterozygote for the c.1186C>T mutation (ii). The single nucleotide transition is indicated by the arrow. The sequence alteration is predicted to cause a substitution of arginine 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).

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