3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and reagents

The chemicals and reagents with analytical grade and highest purity were obtained from the following sources:

GE Healthcare, Sweden

- Acrylamide
- Brilliant Blue R-250
- N, N'-methylene-bis-acrylamide (Bis)
- Plus One dithiothreitol (DTT)
- Plus One sodium dodecyl sulfate (SDS)

Applichem, Germany

- Triton X 100

Applied Biosystems, USA

- Power SYBR ® Green PCR master mix

Bioline, USA

- HyperPAGE Prestained Protein Marker

BDH Laboratory Supplies, UK

- 2-Mercaptoethanol

Calbiochem Biosciences, USA

- Tris (hydroxymethyl) aminomethane

Cambrax Bioscience, USA

- SeaKem ® LE Agarose

Fermentas Life Sciences, USA

- Gene Ruler ™ 100 bp DNA Ladder
- Gene Ruler ™ 100 bp DNA Ladder Plus
- Gene Ruler ™ DNA Ladder Mix
- Thermus acquaticus (Taq) DNA Polymerase (5U/µ) provided with 10X PCR buffer with KCl (100mM Tris-HCl, pH 8.8, 500 mM, 0.8 % Nonidet P40) and 25 mM $MgCl_2$
- PageRuler ™ Prestained Protein Ladder
- 6X DNA loading dye (10 mM Tris-HCl, pH 7.6, 60 mM EDTA, 60 % glycerol, 0.03 % bromophenol blue and xylene cyanol)
- 10 mM deoxyribonucleoside triphosphates mix (dNTP mix)

Invitrogen, USA

- Glycerol

J-Kollin, UK

- Absolute ethanol
- 95 % Ethanol

Mallinckrodt Chemicals, USA

- Methanol (MeOH)

Merck, Germany

- Ethylenediaminetetraacetic acid (EDTA)
- Ethidium bromide
- Glacial acetic acid
- Sodium chloride (NaCl)

Sigma-Aldrich Company, USA

- Dimethylsulfoxide (DMSO)
- Sigma ® water (molecular biology grade)
- Bromophenol Blue
- Phosphate buffered saline
- Ammonium persulfate (APS)
- Guaiacol
- Tris-Hydrochloride (Tris-HCl)

Systerm ChemAR, Poland

- 30 % hydrogen peroxide (H_2O_2-30 %)

Thermo Scientific, USA

- Pierce Protease Inhibitor Cocktail

3.1.2 Kits

Ambion, UK

- DNase I

Applied Biosystems, USA

- High Capacity RNA-to-cDNA Kit

Bio-Rad Laboratories, USA

- Bradford Protein Assay Kit I

Invitrogen, USA

- WesternDot ™ 625 Western blot kit

Qiagen, Germany

- QIAamp® DNA Minikit
- QIAquick® PCR Purification Kit
- MinElute® Gel Extraction Kit
- RNeasy ® Mini
- QIAshredder Homogeniser

3.1.3 Primers

EuroGenTec AIT, Singapore

- Forward and reverse oligonucleotides for *TPO* gene

1st Base, Malaysia

- Forward and reverse oligonucleotides for *TPO*, *TBP*, *Tg*, *TSH-R*, *NIS* genes

3.1.4 Antibodies

Abcam, UK

- Mouse monoclonal MoAb47
- Mouse monoclonal beta-actin antibody Ac-15

3.1.5 Apparatus and instruments

Amersham Pharmacia Biotech, Sweden

- GeneQuant spectrophotometer

Applied Biosystems, USA

- StepOnePlus ™ Real-Time PCR system

Beckman Coulter, USA

- Optima L-100k ultracentrifuge

Biometra, Germany

- PCR thermocycler
- Rocking platform

Bio-rad Laboratories, USA

- Gel documentation system version 2000

Biosan, USA

- Dry block heating thermostat (BioTDB-100)

C.B.S. Scientific, USA

- Dual vertical mini-gel system
- Mini electrophoretic blotting system (EBU-200)

Denver Instrument, USA

- Balance

E-C Apparatus Corporation, USA

- Electrophoretic gel system
- E-C Maxicell ™ EC 360M
- Minicell ® EC 370M

Hwashin Technology, Korea

- Power Sonic 405

Labnet International, USA

- Vortex mixer

Qiagen, Germany

- TissueRuptor

Thermo Electron Corporation, USA

- Sorvall Legend Micro 17 centrifuge

Vilber Lourmat, France

- UV transilluminator

3.2 Methods

3.2.1 Study design

This study was divided into two parts: (1) mutational screening of the *TPO* gene in patients with dyshormonogenetic CH and their family members (2) *in silico* and biochemical analyses of mutant TPO. Our research group had earlier found a previously documented mutation, c.1159G>A, in two siblings with congenital dyshormonogenetic hypothyroidism: CHP41 (index patient, II-4) and his sibling (II-3). In this study, the c.1159G>A mutation was also screened in other family members of CHP41. In addition, 20 unrelated patients with dyshormonogenetic CH and their family members were also recruited for mutational screening. The mutational screening study was carried out involving several molecular techniques such as genomic DNA extraction and quantification, polymerase chain reaction (PCR), PCR products purification and direct DNA sequencing. For the second part of the study, the effects of the mutation (s) found in part (1) on protein function were further investigated by *in silico* analyses using a few computer software products, including Human Splicing Finder (HSF), CLC Sequence Viewer 6.5.2, Clustal X 2.1, Sorting Intolerant From Tolerant (SIFT), Polymorphism Phenotyping version 2 (Polyphen-2), Modeller 9.10, Protein Quality Predictor (ProQ), Discovery Studio 3.0, PSIPRED, Transmembrane Topology (MEMSAT-SVM) and TFbind. The *in silico* findings of one of the mutations (c.2268dup) were further validated with mRNA transcript analysis, Western blot and guaiacol oxidation assays. The effects of the c.2268dup mutation on *TPO* gene expression level was also analysed using quantitative real time polymerase chain reaction (qRT-PCR) and Western blot methods and the ratio of TPO mRNAs spliced variants in normal area of thyroid tissue and those with lesion was calculated. The qRT-PCR method was also used to profile the

expression patterns of the various thyroid hormone-related genes including *Tg, TSH-R* and *NIS in* different types of thyroid tissue.

This study was approved by the University Malaya Medical Central (UMMC) Ethical Committee (Institutional Review Board) in accordance with the ICH-GCP guideline and the Declaration of Helsinki (Ethics number: 654.16). Written informed consent was obtained from patients for their blood to be taken for mutational analysis. Written informed consent was also obtained from parents if patients are below the age of 18 years.

3.2.2 Subjects for the *TPO* mutational screening

The UMMC conducted routine neonatal hypothyroid screening on all live births in the hospital. Babies detected and confirmed to have CH were treated and followed regularly in the Paediatric Endocrine clinic. By age 3 years, all were subjected to radio-imaging studies of the thyroid gland when the treatment was temporarily stopped for 4 - 6 weeks and thyroid function test performed again to confirm permanency of CH.

A cohort of 21 unrelated patients with dyshormonogenetic CH who attended the Paediatric Clinic and their family members were recruited for this study. All except four patients (CHP52, CHP53, CHP59 and CHP60) were diagnosed by newborn screening. Their serum TSH, FT_4 and/or total T_4 values, thyroid status and cognitive assessment (intelligence quotient, IQ) are shown in Table 3.1. Mutational screening was performed on all patients who had raise TSH and low FT_4 and/or total T_4 levels at the time of diagnosis with proven presence of thyroid gland. To confirm that an alteration in the *TPO* gene is due to a disease-causing mutation instead of a polymorphism, a total of

Table 3.1 Profile of patients with dyshormonogenetic CH showing the respective thyroid function and status at the time of diagnosis. ^{99}Tm thyroid scintigraphy was performed in all patients (except CHP49) when they were 3 years when L-thyroxine was temporarily stopped for 4 weeks. The thyroid scan revealed the presence of glands in all tested patients. The CHP49 was below 3 years when the study was carried out.

CH patient (CHP)	Gender	Race	Age of diagnosis	TSH (μIU/ml)	FT_4 (pmol/L)	Total T_4 (nmol/L)	Thyroid scan/ultrasonography	Cognitive assessment (IQ)
16	Female	Chinese	Newborn	26.5	11.3	N/D	Normal	N/A
18	Male	Chinese	Newborn	59.0	13.0	N/D	Goitre	N/A
21	Female	Chinese	3 months	20.6	14.1	N/D	Normal	N/A
22	Male	Chinese	Newborn	33.0	12.2	N/D	Normal	N/A
23	Female	Chinese	Newborn	100.0	3.0	N/D	Goitre	N/A
24	Female	Chinese	Newborn	61.3	13.6	N/D	Normal	N/A
33	Female	Chinese	10 days	120.0	5.0	N/D	Multinodular goitre	Normal
38	Male	Chinese	Newborn	42.6	14.3	N/D	Normal	N/A
40	Male	Chinese	7 weeks	27.8	18.4	N/D	Normal	N/A

Table 3.1 Continued.

CH patient (CHP)	Gender	Race	Age of diagnosis	TSH (μIU/ml)	FT_4 (pmol/L)	Total T_4 (nmol/L)	Thyroid scan/ultrasonography	Cognitive assessment (IQ)
41	Male	Malay	Newborn	57.9	5.4	N/D	Goitre	Normal
45	Male	Chinese	Newborn	217.0	5.0	N/D	Normal	Normal
49	Female	Malay	Newborn	26.4	16.5	N/D	N/A	N/A
51	Male	Chinese	2 weeks	17.7	14.5	N/D	Normal	Normal
52	Female	Indian	12 years	N/A	N/A	N/D	Multinodular goitre	N/A
53	Female	Indian	4 months	160.0	N/A	<13.0	Multinodular goitre	Intellectual disability (50-55)
55	Female	Chinese	7 weeks	>100.0	4.0	N/D	Normal	Intellectual disability (67)
56	Female	Chinese	7 weeks	181.0	N/A	156.0	Normal	Normal
58	Male	Chinese	5 days	37.0	19.9	N/D	Normal	Normal
59	Male	Chinese	3.5 years	>100.0	0.05 (0.04 ng/dl)	N/D	Normal	Below average
60	Female	Indian	5 months	>40.0	2.57 (0.2 ng/dl)	N/D	Normal	N/A

Table 3.1 Continued.

CH patient (CHP)	Gender	Race	Age of diagnosis	TSH (µIU/ml)	FT_4 (pmol/L)	Total T_4 (nmol/L)	Thyroid scan/ultrasonography	Cognitive assessment (IQ)
61	Male	Indian	11 days	91.1	13.3	N/D	Normal	Intellectual disability

N/D: Not done, N/A: Not available

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

Total $T_{4=}$ 60 - 140 nmol/L

Cognitive assessment (IQ) = normal: 85 - 115; below average: 70 - 84; intellectual disability: below 70

100 chromosomes from 50 unrelated healthy individuals were also screened for the same mutation. In addition to the 21 unrelated patients, mutational screening was also carried out in family members of the following patients: CHP41, CHP33, CHP49, CHP51, CHP53, CHP55, CHP58 and CHP59.

3.2.2.1 CHP41 and his family members

CHP41 (II-4) was diagnosed with CH during a routine neonatal screening with serum TSH = 57.9 μIU/ml and FT_4 = 5.4 pmol/L. ^{99}Tm scintigraphy confirmed that he had a thyroid gland but later developed a goitre when he was 9.6 years. Meanwhile, II-3, the elder sister of CHP41 was noted to have a goitre at 12.5 years when the family members were examined and screened for this study. Her serum TSH = 4.8 μIU/ml and FT_4 = 12.4 pmol/L were normal. Both of them had normal growth and development. CH was not detected in the eldest sister, II-1, initially. However, she was later discovered to develop a goitre at the age of 19 years and remained euthyroid. No other congenital anomalies were then detected in CHP41, II-1 and II-3. Another sibling, II-2 and their parents (I-1 and I-2) who are biologically unrelated were all healthy, euthyroid with no congenital anomalies. The profiles of CHP41's family members are summarised in Table 3.2.

3.2.2.2 CHP33 and her family members

CHP33 (III-2) was diagnosed with CH at 10 days after birth with serum TSH = 120.00 μIU/ml and FT_4 = 5.0 pmol/L. Meanwhile, III-1, the elder sister of CHP33 was also detected to have CH at two weeks of life with serum TSH = 96.51 μIU/ml and FT_4 = 2.0 pmol/L. ^{99}Tm scintigraphy confirmed that both patients had thyroid

Table 3.2 Clinical profile of family members of CHP41 with dyshormonogenetic CH.

Index patient	Family members	Relationship	Dyshormonogenetic CH : Symptomatic; Asymptomatic	Thyroid gland (P:presence ; A:absence)	Thyroid gland condition (N: normal; Ab:abnormal)	Others (If any)
CHP41	I-1	Father	Asymptomatic	P	N	-
	I-2	Mother	Asymptomatic	P	N	-
	II-1	Sibling	Asymptomatic	P	Ab	Hypothyroid, goitre
	II-2	Sibling	Asymptomatic	P	N	-
	II-3	Sibling	Symptomatic	P	Ab	Goitre

gland. However, during clinic follow up they developed goitre in their late teenage years and thyroid ultrasonography revealed that both of them had multinodular goitres (MNG). CHP33's human thyroglobulin (hTG) level was 193 ng/ml (reference range 0 - 55 ng/ml) when she was 20 years old. The hTG level of CHP33's sister was unavailable. CHP33 and her sister (III-1) subsequently underwent thyroidectomy at the age of 20 years and 24.5 years, respectively. Histology results of their thyroid glands revealed the presence of follicular adenoma thyroid nodule. No other congenital anomalies were detected and both of them had normal growth and development. Their parents (I-1 and I-2) who are biologically unrelated were all healthy, euthyroid with no congenital anomalies. However, CHP33's maternal grandmother (I-4) was reported to have goitre many years ago and had subsequently undergone thyroidectomy. It was not known what the histology result was. The profiles of CHP33's family members are summarised in Table 3.3.

3.2.2.3 CHP49 and her family members

CHP49 (II-2) was diagnosed with abnormal cord blood serum TSH level = 26.44 μIU/ml and FT_4 = 16.5 pmol/L at birth. She was confirmed to have CH after five days with repeat serum TSH = 25.17 μIU/ml and FT_4 = 16.5 pmol/L. No other congenital anomalies were found in CHP49. Further investigation showed that the patient's brother (II-1) was taking thyroxine treatment for hypothyroidism. He was diagnosed late at three years with short stature with serum TSH = 6.32 μIU/ml and FT_4 = 9.8 pmol/L. ^{99}Tm scintigraphy confirmed that II-1 had thyroid gland. Their parents (I-1 and I-2) are biologically unrelated, healthy, euthyroid with no congenital anomalies. The profiles of CHP49's family members are summarised in Table 3.4.

Table 3.3 Clinical profile of family members of CHP33 with dyshormonogenetic CH.

Index patient	**Family members**	**Relationship**	**Dyshormonogenetic CH : Symptomatic; Asymptomatic**	**Thyroid gland (P:presence ; A:absence)**	**Thyroid gland condition (N: normal; Ab:abnormal)**	**Others (If any)**
CHP33	I-4	Maternal grandmother	Asymptomatic	P	Ab	Goitre
	II-1	Father	Asymptomatic	P	N	-
	II-2	Mother	Asymptomatic	P	N	-
	III-1	Sister	Symptomatic	P	Ab	MNG (follicular adenoma thyroid nodule)

Table 3.4 Clinical profile of family members of CHP49 with dyshormonogenetic CH.

Index patient	Family members	Relationship	Dyshormonogenetic CH : Symptomatic; Asymptomatic	Thyroid gland (P:presence ; A:absence)	Thyroid gland condition (N: normal; Ab:abnormal)	Others (If any)
CHP49	I-1	Father	Asymptomatic	P	N	-
	I-2	Mother	Asymptomatic	P	N	-
	II-1	Brother	Symptomatic	P	N	Delayed growth

3.2.2.4 CHP51 and his family members

CHP51 (III-2) was diagnosed with CH with cord blood serum TSH = 21 μIU/ml and FT_4 = 10.4 pmol/L. A repeat blood for thyroid function test at day 14 of life revealed high TSH (17.7 μIU/ml) and FT4 (14.5 pmol/L) indicating compensated hypothyroidism. When he was three years old his treatment with thyroxine was stopped for four weeks and ^{99}Tm scintigraphy performed which confirmed that he had normal size thyroid gland. A repeat blood for thyroid function test revealed TSH was 47.6 μIU/ml and FT4 was 8.6 pmol/L confirming that he has dyshormonogenetic CH. He had a normal growth and cognitive development. No other congenital anomalies were detected. However, paternal grandfather (I-1) and patient's mother (II-2) had a history of thyroid disease with malignancy development in thyroid gland. The patient's father (II-1), maternal grandmother (I-4) and other siblings (III-1, III-3 and III-4) were all healthy, euthyroid with no congenital anomalies. The profiles of CHP51's family members are summarised in Table 3.5.

3.2.2.5 CHP53 and her family members

CHP53 (II-1) was diagnosed with CH at four months old when she presented with classical signs of hypothyroidism. The serum TSH was more than 160 μIU/ml and total T_4 less than 13.0 nmol/L. Meanwhile, II-3, the younger sister of CHP53 was also detected to have CH at two weeks old when she was screened during her hospital stay for birth asphyxia, with the background history of elder sister with CH. At diagnosis, her free T_4 was 2.1 pmol/L. ^{99}Tm thyroid scintigraphy confirmed that both patients had normal thyroid glands. In the course of their follow up clinic visits, both patients CHP53 and II-3 developed MNG at 11 years and 10 years, respectively. This was confirmed by

Table 3.5 Clinical profile of family members of CHP51 with dyshormonogenetic CH.

Index patient	Family members	Relationship	Dyshormonogenetic CH : Symptomatic; Asymptomatic	Thyroid gland (P:presence ; A:absence)	Thyroid gland condition (N: normal; Ab:abnormal)	Others (If any)
CHP51	I-1	Paternal grandfather	Asymptomatic	P	Ab	Thyroid malignancy
	I-4	Paternal grandmother	Asymptomatic	P	N	-
	II-1	Father	Asymptomatic	P	N	-
	II-2	Mother	Asymptomatic	P	Ab	Thyroid malignancy
	III-1	Brother	Asymptomatic	P	N	-
	III-3	Sister	Asymptomatic	P	N	-

thyroid ultrasonography. Both had negative thyroid antibody. However, the hTG level of II-1 was 137 ng/ml while the hTG level of II-3 was markedly elevated to 1284 ng/ml. Both had FNAB of their goitres, which revealed no evidence of malignancy. Patient CHP53 suffered consequence of late treatment, affecting her growth and cognitive function with IQ of 50 - 55 (normal IQ = 85 - 115). Her younger sister, II-3, had normal physical growth and an IQ of 80 which is slightly below the average IQ range of 85 - 115. No other congenital anomalies were detected. Their parents (I-1 and I-2) were consanguineous (father is the maternal uncle) and the other two proband's siblings (II-2 and II-4) were all healthy, euthyroid with no congenital anomalies. The profiles of CHP53's family members are summarised in Table 3.6.

3.2.2.6 CHP55 and her family members

CHP55 (II-2) was diagnosed with dyshormonogenetic CH at seven weeks old (delayed diagnosis) with blood serum TSH higher than 100 μIU/ml and FT_4 = 4 pmol/L. ^{99}Tm scintigraphy confirmed that she has thyroid gland. CHP55 had a normal growth but intellectual disability (IQ below 67) due to consequence of late treatment. No other congenital anomalies were detected. Her parents (I-1 and I-2) who are biologically unrelated and sibling (II-1) were all healthy, euthyroid with no congenital anomalies. The profiles of CHP53's family members are summarised in Table 3.7.

Table 3.6 Clinical profile of family members of CHP53 with dyshormonogenetic CH.

Index patient	**Family members**	**Relationship**	**Dyshormonogenetic CH : Symptomatic; Asymptomatic**	**Thyroid gland (P:presence ; A:absence)**	**Thyroid gland condition (N: normal; Ab:abnormal)**	**Others (If any)**
CHP53	II-1	Father	Asymptomatic	P	N	-
	II-2	Mother	Asymptomatic	P	Ab	Thyroid malignancy
	III-2	Brother	Asymptomatic	P	N	-
	III-3	Sister	Symptomatic	P	Ab	MNG
	III-4	Sister	Asymptomatic	P	N	-

Table 3.7 Clinical profile of family members of CHP55 with dyshormonogenetic CH.

Index patient	Family members	Relationship	Dyshormonogenetic CH : Symptomatic; Asymptomatic	Thyroid gland (P:presence ; A:absence)	Thyroid gland condition (N: normal; Ab:abnormal)	Others (If any)
CHP55	I-1	Father	Asymptomatic	P	N	-
	I-2	Mother	Asymptomatic	P	N	-
	II-1	Brother	Asymptomatic	P	N	-

3.2.2.7 CHP58 and his family members

CHP58 (II-1) was diagnosed with dyshormonogenetic CH at birth with cord blood serum TSH = 25.7 μIU/ml and FT_4 = 12.1 pmol/L. A repeat blood test at day five of life revealed TSH = 37.7 μIU/ml and FT4 = 19.9 pmol/L confirming compensated hypothyroidism. ^{99}Tm scintigraphy confirmed that he had normal thyroid gland. The repeat blood for thyroid function test when treatment was temporarily stopped at three years revealed TSH = 14.0 μIU/ml and FT_4 = 15.0 pmol/L indicating compensated dyshormonogenetic hypothyroidism No other congenital anomalies were detected. His parents (I-1 and I-2) who are biologically unrelated were all healthy, euthyroid with no congenital anomalies. The profiles of CHP58's family members are summarised in Table 3.8.

3.2.2.8 CHP59 and his family members

CHP59 was detected to have CH at birth with serum TSH higher than 100 μIU/ml and FT_4 = 0.04 ng/dl or 0.5 pmol/L and congenital scoliosis at the age of three and one half years. ^{99}Tm scintigraphy confirmed that he had normal thyroid gland. He had normal growth but was a slow learner. His cognitive assessment (IQ test) result was not available. No other congenital anomalies were detected. Patient's maternal grandmother (I-4), other siblings (III-1 and III-3) and his parents (II-1 and II-2) who are biologically unrelated were all healthy, euthyroid with no congenital anomalies. The profiles of CHP58's family members are summarised in Table 3.9.

Table 3.8 Clinical profile of family members of CHP58 with dyshormonogenetic CH.

Index patient	Family members	Relationship	Dyshormonogenetic CH : Symptomatic; Asymptomatic	Thyroid gland (P:presence ; A:absence)	Thyroid gland condition (N: normal; Ab:abnormal)	Others (If any)
CHP58	I-1	Father	Asymptomatic	P	N	-
	I-2	Mother	Asymptomatic	P	N	-

Table 3.9 Clinical profile of family members of CHP59 with dyshormonogenetic CH.

Index patient	Family members	Relationship	Dyshormonogenetic CH : Symptomatic; Asymptomatic	Thyroid gland (P:presence ; A:absence)	Thyroid gland condition (N: normal; Ab:abnormal)	Others (If any)
CHP59	I-4	Paternal grandmother	Asymptomatic	P	N	-
	II-1	Father	Asymptomatic	P	N	-
	II-2	Mother	Asymptomatic	P	N	-
	III-1	Brother	Asymptomatic	P	N	-
	III-3	Sister	Asymptomatic	P	N	-

3.2.3 Blood sampling and genomic DNA extraction

About 2 ml of venous blood was collected from all subjects into ethylenediamine tetraacetate (K_3 EDTA) tubes and kept at 4 ℃. Genomic DNA was isolated from whole blood mononuclear cells of each sample using QIAamp® DNA Minikit (Qiagen, Germany) according to the manufacturer's protocol. Briefly, about 20 µl of QIAGEN Protease (or proteinase K) was pipetted into the bottom of a sterile 1.5 ml microcentrifuge tube followed by the addition of 200 µl of whole blood. Next, equal volume of buffer AL was added into the tube and the tube was pulse-vortexed for 15 sec. The mixture was then incubated in a thermostat heating block (Biosan, Korea) at 56 ℃ for 10 min followed by a brief spun of the tube. Subsequently, 200 µl of absolute ethanol was added to the sample mixture followed by a 15 sec pulse-vortexing. Afterwards, the tube was briefly spun and the reaction mixture was then pipetted into the center of a QIAamp mini spin column placed in a 2 ml collection tube. The tube was spun for 1 min at 6,000 X g. The tube containing filtrate was discarded while column containing bound DNA was transferred into a new 2 ml collection tube. Five hundred µl of washing buffer, AW1, was added to the column and the tube was centrifuged for 1 min at 6,000 X g. Again, the column was transferred into a new 2 ml collection tube and washed with 500 µl of Buffer AW2 followed by 3 min of centrifugation at 20,000 X g. To ensure a complete removal of the absolute ethanol, the column cap was open and the column was transferred into a new collection tube and spun for 1 min at 20,000 X g. Finally, the column was placed into a sterile 1.5 ml microcentrifuge tube followed by the addition of 200 µl of elution buffer, AE. The tube was incubated for 5 min at room temperature and then spun for 1 min at 6,000 X g. The eluted DNA was stored at -20 ℃ for further analysis. All the centrifugation steps were performed at room temperature using the Legend Micro 17 tabletop centrifuge (Thermo Scientific, USA).

3.2.4 Determination of the yield and quality of the extracted genomic DNA

The yield and quality of the extracted DNA samples were determined spectrophotometrically using GeneQuant spectrophotometer. A dilution factor of 40 (40 X) was set and 80 µl of sterile double distilled water (ddH_2O) was used for calibration. After the calibration, a final volume of 80 µl diluted sample containing 2 µl sample mixed with 78 µl sterile ddH_2O was pipetted into a quartz cuvette and placed in the spectrophotometer. The sample absorbance at 260 nm and 280 nm were measured and concentration was calculated. The purity of the DNA sample was determined by calculating the ratio of absorbance at 260 nm (OD_{260}) to 280 nm (OD_{280}) whereby an OD_{260}/OD_{280} ratio ranging from 1.7 to 1.9 as described in QIAamp® DNA Minikit indicate good quality DNA. The estimated concentration of the extracted DNA was calculated as shown below:

Concentration (µg/ml) = A260 reading x Dilution factor x 50 µg/ml

Assuming that A_{260} of 1.0 = 50 µg/ml pure dsDNA

3.2.5 Mutational analysis of the *TPO* gene

Mutational analysis in all 17 exons of the *TPO* gene was carried out in 13/21 unrelated CH patients (CHP33, CHP38, CHP41, CHP49, CHP51, CHP52, CHP53, CHP55, CHP56, CHP58, CHP59, CHP60 and CHP61). Meanwhile, the other 8/21 unrelated Malaysian-Chinese patients diagnosed with dyshormonogenetic CH were screened for a common mutation, c2268dup (Niu *et al.*, 2002), in exon 13 only due to limited amount of DNA samples available. To confirm that an alteration in the *TPO* gene is due to a disease-causing mutation instead of a polymorphism, family members

of the index patients and a total of 100 chromosomes from 50 unrelated healthy individuals were also screened for the same mutation.

3.2.5.1 Polymerase chain reaction (PCR)

The *TPO* gene was PCR-amplified with primers (Rivolta *et al.*, 2003; Wu *et al.*, 2002) covering all the exons (1 - 17) including the hotspot mutation regions, exons 8, 9, 10, 12 and 14. A 50 µl PCR reaction tubes contained 100 - 250 ng of genomic DNA, 1X Taq buffer with KCl, 2.5 mM of MgCl, 200 µM of dNTP, 8 % dimethylsulfoxide (DMSO), 1 unit of *Taq* DNA polymerase (Fermentas, USA) and 20 pmol of each forward and reverse primers were prepared in a master mix. A master mix that contained all reagents but without *Taq* DNA polymerase, primers and genomic DNA was prepared first, *Taq* DNA polymerase was then added into the master mix and mixed well. The mixture in the master mix tube was then transferred into PCR tubes which contained primers and genomic DNA template. The PCR tubes were vortexed briefly, spun down, and placed in a pre-heated PCR thermal cycler (Biometra, Germany). A negative control was included in every reaction to confirm the absence of contamination. Oligonucleotide sequences and their respective annealing temperature are shown in Table 3.10. The PCR protocol was as follows: initial denaturation step at 94 ℃ for 2 min, followed by 35 cycles of amplification with denaturation step at 95 ℃ for 30 sec, primer annealing step at suitable temperature for the respective exon for 30 sec and elongation step at 72 ℃ for 1 min. The program was completed with final extension at 72 ℃ for 10 min and, lastly, held at 4 ℃. The PCR products were stored at -20 ℃ or analysed on gel electrophoresis.

Table 3.10 Sequence of primers used for PCR amplification of exons 1 to 17 of the *TPO* gene, size of PCR products and the annealing temperature needed for each pair of primers. All primer sequences were sourced from Rivolta *et al*. (2003) except for the exon 10 which was sourced from Wu *et al*. (2002). Exon sequences are in uppercase; intron sequences are in lower case letters.

Exon	PCR primers	Fragment size (bp)	Annealing temperature (℃)	Source
1	5’-ctgtccccacgaagaac-3’ (1F) 5’-gctgagagacgccaccc-3’ (1R)	305	55	Rivolta *et al.*, 2003
2	5’-gtagaggctgcgtggag-3’ (2F) 5’-gtgactctcaggagcta-3’ (2R)	306	55	Rivolta *et al.*, 2003
3	5’-ggcatcaccgcagcaag-3’ (3F) 5’-cacacgtgtgtggatgt-3’ (3R)	259	55	Rivolta *et al*., 2003
4	5’-tgccattttcctcatca-3’ (4F) 5’-catcctgcttggtctca-3’ (4R)	523	44	Rivolta *et al*., 2003
5	5’-atggtttcctatttttcacagAT-3’ (5F) 5’-cggggaggccaaggaca-3’ (5R)	294	55	Rivolta *et al*., 2003

Table 3.10 Continued.

Exon	PCR primers	Fragment size (bp)	Annealing temperature (℃)	Source
6	5’-ggccccacttattctcc-3’ (6F) 5’-ttccctccctcagcatc-3’ (6R)	296	55	Rivolta *et al*., 2003
7	5’-gaaccacaccaggaagt3’ (7F) 5’-tgggaataggacaaga-3’ (7R)	411	44	Rivolta *et al.*, 2003
8	5’-tcgtcgccggcctcgaactt-3’ (8F) 5’-ggagagagaagccacgatgc-3’ (8R)	690	55	Rivolta *et al.*, 2003
9	5’-cagctgaggcccttattaca-3’ (9F) 5’-ggaccgcactcactcacCTC-3’ (9R)	415	55	Rivolta *et al*., 2003
10	5’- ctagaactgagccaagagc -3’ (10F) 5’-tgtgcaagggaaggaactg-3’ (10R)	245	55	Wu *et al*., 2002

Table 3.10 Continued.

Exon	PCR primers	Fragment size (bp)	Annealing temperature (℃)	Source
11	5’-aaaccctgcagcctctccc3’ (11F) 5’-cgtgaaggaagacgctctg-3’ (11R)	297	55	Rivolta *et al*., 2003
12	5’-ttctccatgcactgtgacc-3’ (12F) 5’-atgcacgtgctgtaacgtgg-3’ (12R)	296	55	Rivolta *et al*., 2003
13	5’-acagggacgttggtgtgtgg-3’ (13F) 5’-tcagaagcacctttggcg3’ (13R)	344	55	Rivolta *et al.*, 2003
14	5’-tgcagccgcttcctctcacg-3’ (14F) 5’-gatggtgattgacagttgcc-3’ (14R)	271	55	Rivolta *et al.*, 2003
15	5’-agactcaggcaggacaacc-3’ (15F) 5’-gcttcattgcagccatgtcc-3’ (15R)	249	55	Rivolta *et al*., 2003

Table 3.10 Continued.

Exon	PCR primers	Fragment size (bp)	Annealing temperature (℃)	Source
16	5’-tgccggaccctctcccgataa-3’ (16F) 5’-gacacccagatcctgtccaa-3’ (16R)	236	55	Rivolta *et al*., 2003
17	5’-aagaaggatggctcatctcg-3’ (17F) 5’-CTGCTGATTTCCGATTTGCC-3’ (17R)	285	55	Rivolta *et al*., 2003

3.2.5.2 Agarose gel electrophoresis

Different concentrations of agarose gel (1 %, 2 % or 3 %, measured in w/v) were used to separate PCR products in the size range of 100 - 3000 bp. One percent agarose gel was used for those in the size range of 1000 - 3000 bp while 2 % agarose gel was used for those in the size range of 200 - 1000 bp. Three percent gel was only prepared for separation of very tiny fragments. Gels were prepared in 1 X TAE buffer. The solution was heated in a microwave until all agarose powder was completely dissolved. Then 0.2 ug/ml of EtBr was added to the mixture and mixed well. The mixture was cooled to about 50 ℃ and was carefully poured into a Minicell (1 % and 2 % agarose gels) or Maxicell (3 % agarose gel) casting tray with pre-inserted comb and rubber casting dams to devoid any air bubbles especially around the comb teeth. The gel was allowed to solidify completely at room temperature. Once the gel was solidified, the comb and rubber casting dams were removed and the tray was placed in electrophoresis chamber covered with 1 X TAE buffer. Five volume of DNA sample was mixed with one volume of 6 X loading dye (Fermentas Life Sciences, USA) and loaded into the wells. One ul of DNA ladder (GeneRuler™ 100 bp DNA Ladder or GeneRuler™ DNA Ladder Mix, Fermentas Life Sciences, USA) was also loaded into one of the wells as a standard to estimate the size of the PCR products. Electrophoresis process was carried out at 90 V for about 50 min until the loading dye moved to about ¾ of the gel. For a 3 % agarose gel, electrophoresis process was carried out at 60 V for 8 h. The resulting DNA bands were visualised under ultra violet (UV) light (300 nm wavelength) using a gel documentation system (Bio-Rad Version 2000).

3.2.5.3 Purification of the PCR product

Purification of the PCR product was performed using either QIAquick® PCR Purification Kit or MinElute® Gel Extraction Kit (Qiagen, Germany).

3.2.5.3.1 Purification of the PCR product by QIAquick® PCR Purification Kit (Qiagen, Germany)

PCR products were purified using QIAquick® PCR Purification Kit (Qiagen, Germany) following the manufacturer's instruction. Five volumes of PBI buffer was added to 1 volume of the PCR products and mixed by pipetting producing a yellow color mixture. The mixture was then transferred into a QIAquick spin column placed in a 2 ml collection tube. The column was spun for 45 - 60 sec and the flow-through was discarded. The column was placed back into the same collection tube. Next, the washing step was carried out by adding 750 µl of PE buffer into the column followed by 45 - 60 sec of centrifugation. The flow-through was discarded and the column was placed back into the same collection tube. Finally, the column was transferred into a new 1.5 ml microcentrifuge tube followed by the addition of 30 µl of elution EB buffer (10 mM Tris-Cl, pH 8.5). The column was incubated at room temperature for 1 min followed by 1 min of centrifugation at 17,900 X g. All of the centrifugation steps were performed at room temperature using the Legend Micro 17 tabletop centrifuge (Thermo Scientific, USA).

3.2.5.3.2 Purification of the PCR product by MinElute® Gel Extraction Kit (Qiagen, Germany)

To purify the PCR products from the agarose gel, MinElute® Gel Extraction Kit (Qiagen, Germany) was used. The PCR fragment from the agarose gel was excised with a clean, sharp scalpel. The gel slide was then weighed in a 2 ml clean microcentrifuge tube. Three volumes of QG buffer were added to one volume (mass) of excised gel as recommended in the protocol. The mixture was incubated for 10 min at 50 ℃ until the gel slice has completely dissolved. To help dissolve the gel, the tube was vortexed every 2 - 3 min during the incubation. Next, one gel volume of isopropanol was added to the sample and mixed by inverting the tube several times. The mixture was then transferred into the MinElute column sitting in a 2 ml collection tube. The column was spun for 1 min allowing the DNA to bind on the silica membrane. The flow-through was discarded and the column was placed back into the same collection tube. Next, 500 µl of the QG buffer was added to the spin column and spun for 1 min. The flow-through was discarded and the column was placed back into the same collection tube. Next, the washing step was carried out by adding 750 µl of PE buffer into the column followed by 1 min of centrifugation. The flow-through was discarded and the column was spun for additional 1 min. Finally, the column was transferred into a new 1.5 ml microcentrifuge tube followed by the addition of 15 µl of elution EB buffer (10 mM Tris-Cl, pH 8.5). The column was incubated at room temperature for 1 min followed by 1 min centrifugation at 17,900 X g. All centrifugation steps were performed at room temperature using the Legend Micro 17 tabletop centrifuge (Thermo Scientific, USA).

3.2.5.4 Analysis of the purified PCR products

After purification, the purified PCR products were analysed using agarose gel electrophoresis as described in Section 3.2.5.2 to confirm the presence of only the desired band.

3.2.5.5 DNA sequencing of the PCR products

Direct DNA sequencing of the *TPO* gene was carried out using ABI Prism Gene Sequencer, Model 3100, version 3.7 (Research Biolabs, Singapore). The PCR products were sequenced using the same *TPO* primers used in the PCR amplification. The volume and concentration of a PCR product required for each sequence were 10 µl and 30 ng/µl, respectively. The nucleotide sequence obtained was compared to the published sequence available at the National Center for Biotechnology Information (NCBI) gene bank database.

3.2.6 Designing new primer pairs

Additional primer pairs listed in Tables 3.11 and 3.12 were specially designed using Primer3Plus (http://biotools.umassemed.edu/cgi-bin/primer3plus/primer3plus.cgi) software. All primers were designed with 20 - 22 bp in length and had approximately 50 % GC content to amplify products ranging from 200 - 500 bp. The nucleotide sequences of the primer pairs were then referred to the NCBI human *TP*O nucleotides sequence (NCBI access number-genomic: NG_011581.1 or mRNA: NM_000547).

3.2.7 Assessing the significance of nucleotide sequence alterations in the *TPO* gene on protein functions using computational methods

Mutations found were further analysed *in silico* for putative functional effects using several software: Human Splicing Finder (HSF) (http://www.umd.be/HSF/), CLC Sequence Viewer 6.5.2, Clustal X 2.1, Sorting Intolerant From Tolerant (SIFT) (http://sift.jcvi.org/www/SIFT_enst_submit.html), Polymorphism Phenotyping v2 (Polyphen-2) (http://genetics.bwh.harvard.edu/pph2/), Modeller 9.10, Protein Quality Predictor (ProQ) (http://www.sbc.su.se/~bjornw/ProQ/ProQ.html), Discovery Studio 3.0, PSIPRED and Transmembrane Topology (MEMSAT-SVM) (http://bioinf.cs.ucl.ac.uk/psipred/), TFbind (http://tfbind.hgc.jp/).

3.2.7.1 Assessing the potential impact of *TPO* sequence alterations on splicing activity using HSF algorithm

The Human Splicing Finder (http://www.umd.be/HSF/) is online software to predict the effects of a mutation on splicing signals or to identify splicing motifs in any human gene sequence. It was used to identify the location of potential splice sites on *TPO* sequence. The ESE Finder matrices analysis for SRp40, SC35, SF2/ASF, SF2/ASF, IgM/BRCA1, and SRp55; and exonic splicing enhancer (ESE) motifs from HSF (ESE-HSFs) analysis for Tra2 and 9G8, were used to analyse the effects of the mutation/polymorphism on binding site sequences of the above putative splicing regulatory proteins.

3.2.7.2 Multiple amino acid sequence alignment

The conservation of an amino acid was examined using multiple sequence alignments constructed with either CLC Sequence Viewer 6.5.2 or Clustal X 2.1. The TPO protein sequence (UniProt P07202) was aligned with those of mouse (UniProt P35419), rat (UniProt P14650), pig (UniProt P09933), canfa (UniProt Q8HYB7) and chick (UniProt Q2WBI1). The importance of a mutated amino acid residue was determined by the conservation level at its position.

3.2.7.3 SIFT and PolyPhen-2

The SIFT (http://sift.jcvi.org/) (Ng & Henikoff, 2001) and PolyPhen-2 (http://genetics.bwh.harward.edu/pph2/) (Adzhubei *et al.*, 2010) are web-based algorithm that predict how amino acid substitutions will affect protein function. Calculations are based on amino acid homology or the mapping of the substitution site to known protein 3-dimensional structures. Amino acid substitution results are reported as "tolerated" or as "intolerance" for SIFT while results are given as "benign", "possibly damaging", "probably damaging" or "unknown" for Polyphen.

3.2.7.4 PSIPRED

The PSIPRED Protein Structure Prediction Server (http://bioinf.cs.ucl.ac.uk/psipred/) (Buchan *et al.*, 2010) was used to predict the secondary structure (PSIPRED V3.0) and transmembrane topology (MEMSAT-SVM) (Nugent & Jones, 2009) of the TPO protein.

3.2.7.5 Tertiary structure prediction

Since the crystal structure of human TPO is not available, a homology model of the wild type human TPO was generated. Using the online protein-protein BLAST program (Altschul *et al.*, 1990; Mount, 2007) from NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi), the human TPO protein was found to have the highest homology to that of sheep lactoperoxidase where the crystal structure is available (PDB id: 2IKC). Thus, the homology model was generated with 2IKC as a template using Modeller 9.10 software (Sali & Blundell, 1993). For structural comparison, a homology model of the mutated human TPO proteins was also generated. The quality of the generated TPO models was further assessed by ProQ algorithm (http://www.sbc.su.se/~bjornw/ProQ/ProQ.html) (Wallner & Elofsson, 2003). In order to increase the reliability and performance of the ProQ algorithm, the predicted secondary structure from PSIPRED algorithm for each model was included in the assessment. Structure with LGscore > 1.5 and MaxSub > 0.1 is considered as correct model. The generated models were further visualised and analysed using Discovery Studio 3.0 algorithm.

3.2.7.6 The search for the binding site for the transcription factors: TATA box, CAAT box, GC box in upstream region of the *TPO* gene

The TATA box, CCAAT box, GC box were searched in a region 200 bp upstream from the *TPO* gene transcription site using TFBind algorithm (Tsunoda & Takagi, 1999). The analysed sequence of the *TPO* gene was referred to the NCBI human *TPO* nucleotides sequence (NCBI access number-genomic: NG_011581.1, nucleotide number: 4799 to 4998).

3.2.8 TPO mRNA transcript analysis and TPO protein enzymatic analysis in CHP33 (III-2) and her affected sister (III-1) with c.2268dup (p.Glu757X) mutation

Human thyroid tissues specimen from CHP33 and her sister who had undergone thyroidectomy were kindly provided by Dr. Pathmanathan Rajadurai (with the patients' consent) from Sime Darby Medical Center. A piece of normal tissue was taken near the colloid nodules whereas the abnormal tissue was taken from the thyroid lesion. Tumours were diagnosed histopathologically and no evidence of malignancy was detected. Tissues taken during surgery were immediately kept frozen in dry ice and stored at -80 ℃ until further processing.

3.2.8.1 Total cellular RNA isolation

Total cellular RNA (tcRNA) isolation was performed using the RNeasy ® Mini kit (Qiagen, Germany) according to the manufacturer's protocol. First, 30 mg of frozen thyroid tissues were excised with a clean, sharp scalpel. The weighed frozen tissue was homogenised by using TissueRuptor (Qiagen, Germany) in 600 µl of buffer RLT. The lysate was then pipetted into the centre of a QIAshredder homogeniser placed in a 2 ml collection tube and the tube was subjected to 2 min centrifugation at a maximum speed. The column was discarded while the tube containing the filtrate was kept. The lysate was transferred to a new microcentrifuge tube and spun 3 min at full speed. The supernatant was then carefully transferred into a new microcentrifuge tube. Six hundred µl of 70 % ethanol was added to the homogenised lysate and the mixture was briefly mixed by pipetting. Afterwards, the reaction mixture was pipetted into the center of an RNeasy spin column placed in a 2 ml collection tube. The tube was spun for 15 sec at a speed of 10,000 X g. The tube containing the filtrate was discarded while the column

containing bound RNA was transferred into a new 2 ml collection tube. Seven hundred µl of washing buffer, RW1, was added to the column and centrifuged for 15 sec at speed of 10,000 X g. The column was then transferred into a new 2 ml collection tube and washed with 500 µl of buffer RPE. The tube was then spun for 15 sec at speed of 10,000 X g. Next, the filtrate was discarded and the column was placed back to the same collection tube. Again, 500 µl of buffer RPE was added to the column and spun for 2 min at maximum speed. To ensure complete removal of the absolute ethanol, the column cap was open and transferred into a new collection tube and spun for 1 min at 17,900 X g. Finally, the column was placed into a sterile 1.5 ml microcentrifuge tube followed by the addition of 30 µl of RNase-free water. The tube was then spun for 1 min at speed of 10,000 X g. The eluted tcRNA was stored at -80 ℃ for further analysis.

3.2.8.2 Elimination of genomic DNA from RNA samples by DNase I treatment

The extracted tcRNA samples were further purified using DNase I. First, 1/20th volume of 20 X DNase buffer and 1 µl of DNase I (8 U/µl) were added to the tcRNA sample. The mixture was then incubated in a thermostat heating block (Biosan, Korea) at 37 ℃ for 30 min. Subsequently, 20 % equal volume of DNase Inactivation reagent was added into the mixture followed by 30 sec of pulse-vortexing. The tube was incubated for 2 min at room temperature and the mixture was mixed briefly using a vortex during the incubation period. Lastly, the tube was spun for 1 min at 10,000 X g and the supernatant containing tcRNA was transferred into a new RNase-free tube and stored at -80 ℃ for further analysis. All the centrifugation steps were performed at room temperature using Legend Micro 17 tabletop centrifuge (Thermo Scientific, USA).

3.2.8.3 Determination of the yield, quality and integrity of tcRNA

The yield and quality of the extracted tcRNA were determined spectrophotometrically using GeneQuant as described in Section 3.2.4. The concentration of the tcRNA was estimated based on the assumption that an A_{260} reading of 1.0 is equivalent to 40 µg/ml single-stranded RNA. The purity of the RNA sample was determined by calculating the ratio of absorbances at OD_{230}/OD_{260} and OD_{280}/OD_{260}. Both readings range from 1.8 to 2.1 as described in RNeasy ® Mini kit indicate pure RNA.

The integrity of tcRNA sample extracted from the thyroid tissues was further analysed by gel electrophoresis. One part of tcRNA sample was mixed with two parts of RNA loading buffer (95 % formamid, 20 mM EDTA, 0.05 % bromophenol blue and 0.05 % xylene cyanol). The mixture was heated at 80 ℃ for 5 min in a thermostat heating block (Biosan, Korea) prior to loading. Electrophoresis was carried as described in Section 3.2.5.2. The resulting RNA bands were visualised under UV light (300 nm wavelength) using a gel documentation system (Bio-Rad Version 2000).

3.2.8.4 Reverse transcription of tcRNA to cDNA

TcRNA isolated from the thyroid tissues was reverse-transcribed to complimentary DNA (cDNA) using the High Capacity RNA-to-cDNA Kit (Applied Biosystems, USA) according to the manufacturer's protocol. One microgram of tcRNA was reverse-transcribed in a 20 µl reaction mixture containing 10 µl of 2 X RT buffer and 1 µl of 20 X RT enzyme mix. The mixture was mixed briefly using a vortex, spun down, and placed in a pre-heated PCR thermal cycler (Biometra, Germany). The reverse

transcription of tcRNA to cDNA protocol was as follows: the sample was first heated at 37 ℃ for 60 min, followed by completion step with denaturation at 95 ℃ for 5 min and held at 4 ℃.

3.2.8.5 Confirmation of the *in silico* HSF analysis result of the c2268dup mutation

Messenger RNA transcript analysis via reverse transcription-polymerase chain reaction (RT-PCR) method was carried out to confirm the potential splice site prediction analysis result which was performed earlier using *in silico* method. Primer pairs listed in Table 3.11 were used for the RT-PCR amplification. PCR and gel electrophoresis were performed as described in Sections 3.2.5.1 and 3.2.5.2 except that cDNA of CHP33 and her sister instead of genomic DNA was used as a template in this study. Additional cDNA samples prepared from thyroid tissue of c.2268dup mutation-free individuals were used as a positive control in this analysis. Finally, the PCR products were purified, analysed, and then sent for cDNA sequencing as described in Sections 3.2.5.3, 3.2.5.4 and 3.2.5.5, respectively.

3.2.8.6 Genetic analysis of the upstream region of exon 13 of the *TPO* gene in CHP33

The upstream region of exon 13 of the *TPO* gene (141 bp from the acceptor splice site of exon 13, c.2216-141) in CHP33 was screened for alterations. The analysis was carried out by using PCR method as described in Section 3.2.5.1 with forward primer, 5’- gctcagtgagtgaccacagc-3’, and reverse primer, 5'-CTGGAAATCCCATCCTTCCT-3’ at annealing temperature of 55 ℃ . The resulting

Table 3.11 Nucleotide sequence of PCR primers and the size of PCR products for mRNA transcript analysis of the *TPO* gene. Exon sequences are in uppercase; intron sequences are in lower case letters. The "E12/E13F and E13R" and "E12/I12F and E13R" primers pairs were also used in Section 3.2.9.2.3.

Amplifying region	Exon (E) Intron (I), Forward (F) Reverse (R)	PCR primers	Fragment size (bp)	Annealing temperature (℃)
c.18 to c.688	E2F E7R	5'GCTGTCTGTCACGCTGGTTA3' 5'GGAGGTCAGAATAGCGGTCA3'	671	53
c.613 to c.1415	E7F E9R	5'GTCCGGGAGGTGACAAGAC3' 5'CCTTCATAGGGACCCACGTA3'	803	53
c.613 to c.1100	E7F E8R	5'GTCCGGGAGGTGACAAGAC3' 5'ACGAAGGGCAGGTAGGCG3' (Rivolta *et al.*, 2003)	488	53
c.979 to c.1415	E8F E9R	5'TCCACCGTGTATGGCAGCTC 3' (Rivolta *et al.*, 2003) 5'CCTTCATAGGGACCCACGTA3'	437	53
c.1572 to c.2070	E9F E12R	5'CAGCCCATGGACATTACTCC3' 5'GGAGTGCTTCTCCAGCTCAC3'	499	53
c.2071 to c.2370	E12F E13R	5'-CTGTCTCGGGTCATCTGTGA-3' 5'-CTGGAAATCCCATCCTTCCT-3'	300	55
c.2207 to c.2370	E12/E13F E13R	5'-TTCCTCAAGACGACAAGTGTG-3' 5'-CTGGAAATCCCATCCTTCCT-3'	164	55
c.2203 to c.2215…c.2216-34 to c.2370	E12/I12F E13R	5'-ACCTTTCCTCAAGtttgactac-3' 5'-CTGGAAATCCCATCCTTCCT-3'	202	55
c.2351 to c.2600	E13F E15R	5'AGGAAGGATGGGATTTCCAG3' 5'GAGGTGAGACCTGCGAAGC3'	250	53
c.2551 to c.2793	E15F E17R	5' CCTCGGGTGACTTGGATCT3' 5' TCTCGGCAGCCTGTGAGTATC3'(Ferrand *et al.*, 2003)	261	53

band (296 bp) was visualised, purified, analysed, and then sent for sequencing as described in Sections 3.2.5.2, 3.2.5.3, 3.2.5.4 and 3.2.5.5, respectively.

3.2.8.7 Microsomal proteins isolation

The frozen thyroid tissue specimens were weighed and washed with ice-cold phosphate buffered saline (PBS) containing Pierce Protease Inhibitors Cocktail (Thermo Scientific, USA). The washed tissues were homogenised using TissueRuptor (Qiagen, Germany) and then centrifuged for 15 min at 3,000 X g in Sorvall Legend Micro 17 (Thermo Electron Corporation, USA) at 4 ℃ to remove nuclei and cytoskeletal components. The supernatants were subsequently ultracentrifuged in an ultracentrifuge machine, Optima L-100k (Beckman Coulter, USA), at 100,000 X g for 2 h at 4 ℃. The pellet were collected and dissolved in the lysis buffer (50 mM Tris-HCl, pH7.3; 150 mM NaCl; 5 mM EDTA; 10 % glycerol (w/v); 1 % Triton X-100 (w/v), protease inhibitors) (McDonald & Pearce, 2009). Ultrasonication method (Power Sonic 405 by Hwashin Technology, Korea) was used to improve the yield of the extracted protein.

3.2.8.8 Bradford protein assay

The concentration of microsomal protein was estimated using Bradford Protein Assay Kit I (Bio-Rad, USA) with bovine serum albumin (BSA) as the protein standard. Prior to the assay, BSA stock (2 mg/ml) was diluted to 1 mg/ml in double distilled water (ddH_2O). Two hundred µl of Bradford dye reagent was pipetted into each well of the 96-well dish. The BSA stock was added to the wells in triplicate with a final concentration range of 1 mg/ml - 10 mg/ml. One µl of each protein sample was added to the wells in triplicate. The mixture was incubated in a 96-well dish for 5 min at

room temperature and the absorbance was read at 595 nm (OD_{595}). A BSA standard curve was generated using Microsoft Excel and the protein concentration for each sample was calculated from the curve.

3.2.8.9 SDS-PAGE and Western blot

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot were carried out to detect the presence of TPO protein in the tissue specimens.

3.2.8.9.1 Gel apparatus assembly for SDS-PAGE

Two glass plates of 10 cm X 10 cm and 10 cm X 8 cm were cleaned with 70 % ethanol and dried completely. A rubber slab was then inserted along the side of edge of the glass plate (10 cm X 10 cm). Spacers were aligned beside the rubber slab and short plate was placed on top of a spacer plate to form a glass sandwich. The glass sandwich was clamped firmly at left, right and bottom sides to avoid any leakage. Then, ddH_2O was loaded into the assembled gel apparatus to ensure no leakage. The water was then drained off and the apparatus was left to dry.

3.2.8.9.2 Preparation of polyacrylamide gel (PAGE)

Two pieces of 12 % separating gel were prepared by combining all these reagents: 6 ml of 30 % acrylamide / 0.8 % Bis (30 %: 0.8 %), 3.75 ml of 4 X Tris. HCl / SDS, pH 8.8, 5.25 ml of ddH_2O, 0.05 ml of 10 % ammonium persulfate (APS) and 0.01 ml of tetramethylethylenediamine (TEMED, added last). The separating gel

mixture was pipetted into the assembled glass sandwich until it was about 2 cm from reaching the top of the short plate. Prior to polymerisation, a small layer of ddH_2O was added on top of the gel to straighten the level of the gel and to remove unwanted air bubbles that may be present. The water was then drained off when the gel was completely polymerised.

A mixture solution for stacking gel which contained 0.65 ml of 30 % acrylamide / 0.8 % Bis (30 %:0.8 %), 1.25 ml of 4 X Tris. HCl / SDS, pH 6.8, 3.05 ml of ddH_2O, 0.025 ml of 10 % ammonium persulfate (APS) and 0.005 ml of TEMED (added last) was prepared and poured on top of both of the separating gels. A 10-well teflon comb was carefully inserted with slight tilt into the gel sandwich to avoid trapping bubbles under the comb teeth. The gel was allowed to set at room temperature for 1 h. After the gel polymerised, the comb was gently removed and the gel apparatus was assembled onto the electrophoresis buffer tank. The wells were flushed with 1 X SDS-PAGE running buffer (25 mM Tris, 192 mM glycine, 0.1 % SDS) to remove excess gel residuals and air bubbles.

3.2.8.9.3 Sample preparation for SDS-PAGE

Twenty μg of microsomal fraction extract for each sample and 300 ng of positive control (recombinant fragment of human thyroid peroxidase from Abcam, product code: ab73765, UK) were incubated in Laemmli loading buffer. Five volumes of sample was mixed to one volume of 6 X Laemmli Buffer and boiled for 5 min. To verify the antibody specificity of binding antigen, same amount of microsomal protein extracted (Section 3.2.8.8) from a rat thyroid specimen (as a gift from Dr. Azlina

Abdul Aziz, Department of Molecular Medicine, University of Malaya) was prepared by using the same method and included in the experiment.

3.2.8.9.4 Electrophoresis

Both polyacrylamide gels in the electrophoresis tank were submerged in 1 X SDS-PAGE running buffer. Twenty µl of the samples and 7 µl of the HyperPAGE Prestained Protein Marker or 5 µl of the PageRuler ™ Pre-stained Protein Ladder were loaded into the wells of the each gel. Electrophoresis was performed at room temperature with a constant voltage of 100 V. The electrophoresis was stopped when the blue dye has migrated to the bottom of the gel.

3.2.8.9.5 Staining of proteins in gels with Coomassie stain

One of the gels was stained with Coomassie blue to ensure the proteins have migrated uniformly and evenly. The PAGE gel was incubated in pre-heated staining solution (0.1 % Coomassie Brilliant Blue R-250, 50 % methanol (MeOH) and 10 % glacial acetic acid) for 10 min. The stained gel was subsequently transferred into destaining solution (40 % MeOH and 10 % glacial acetic acid) and incubated for overnight with gentle agitation. The destained gel was washed with ddH_2O. The resulting protein bands were visualised under white light using a gel documentation system (Bio-Rad Version 2000).

3.2.8.9.6 Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets

The separated proteins from the unstained gel were transferred to a membrane by using wet transfer method in a mini electrophoretic blotting system (C.B.S. Scientific, USA**)**. Prior to blotting, sponges, papers and polyvinylidene fluoride (PVDF) membrane were soaked in pre-cooled tris-glycine buffer for 10 min. The gel and membrane were arranged in the following order: sponge / paper / gel / PVDF membrane / paper / sponge, and were then clamped tightly together to ensure no air bubbles were formed in between the gel and the membrane. The sandwiched gel was held using a cassette and inserted vertically into a transfer tank containing pre-cooled tris-glycine buffer. Electrophoresis was performed with a constant voltage of 100 V for 1 h at 4 ℃. After the transfer was completed, the moist membrane was washed with ddH_2O to remove excess gel residuals and then placed on a WesternDot ™ staining dish (Invitrogen, USA).

3.2.8.9.7 Immunodetection

Immunodetection analysis was performed using WesternDot ™ 625 Western blot kits (Invitrogen, USA) according to the manufacturer's protocol. First, 8 ml of WesternDot ™ blocking buffer (Invitrogen, USA) was pipetted into the staining disc containing membrane and incubated for 60 min on a shaker set at 1 revolution/second. Mouse monoclonal MoAb47 primary antibody (Abcam, UK) was diluted in 8 ml of 1 X washing buffer at the dilution of 1:2000. The blocking buffer in the staining disc was discarded and replaced with diluted primary antibody. The primary antibody was decanted after a 60 min incubation period on a shaker. The membrane was washed for 5

min with 15 ml of 1 X washing buffer and the buffer was discarded. The washing step was repeated twice. During the last wash, secondary antibody solution was prepared by diluting 4 µl of Biotin-XX-Goat anti-mouse into 8 ml of 1 X washing buffer. The prepared diluted secondary antibody was pipetted into the staining dish and incubated for 60 min. After incubation, the secondary antibody solution in staining disc was discarded and replaced with washing buffer. The washing step was carried out and repeated twice as described above. During the last wash, Qdot ™ 625 streptavidin conjugate was prepared by diluting 4 µl of Qdot ™ 625 streptavidin conjugate into 8 ml WesternDot™ blocking buffer. The membrane in staining disc was incubated with Qdot ™ 625 streptavidin for 60 min and then decanted. After incubation, the washing step as described above was repeated for 3 times. The membrane was proceeded with final washed by using 20 ml sterile milliQ water and visualised under UV light (300 nm wavelength) using a gel documentation system (Bio-Rad Version 2000). After examination, the whole immunodetection process was repeated using the same membrane but different primary antibody (loading control, mouse monoclonal beta-actin antibody Ac-15).

3.2.8.10 Guaiacol oxidation assay

Microsomal fraction extract prepared from patients' thyroid gland tissues (CHP33 and her sister), as described in Section 3.2.8.7, were used for the enzymatic assay. Microsomes prepared from rat thyroid gland tissue were used as a positive control. Twenty µg of microsomal fraction extract for each sample were added to 1 ml of 40 mM guaiacol (Sigma-Aldrich, USA) and 67 mM sodium phosphate buffer, pH 7.5. The enzymatic reaction was initiated by adding H_2O_2 (final concentration, 0.25 mM). The absorbance was measured at 470 nm (OD_{470}) at every second for 3 min at room

temperature. The blank was set without adding H_2O_2. In addition, a TPO-free negative control was run that consists of only buffer and H_2O_2 was also included.

3.2.9 Quantification for expression of TPO in thyroid tissue of CHP33 (III-2) and her affected sister (III-1)

3.2.9.1 Relative quantification of TPO proteins on Western Blots

The expression levels of TPO in normal and lesion areas of thyroid tissue of CHP33 and her sister as previously analysed in Section 3.2.8.9.7 were measured and compared by densitometry scanning method using Image-J software (http://rsbweb.nih.gov/ij/). Expression of TPO in normal and lesion areas of the thyroid tissue was normalised to the expression of beta-actin loading control and compared. Secondly, the total expression of TPO protein in CHP33 was compared to her sister after beta-actin loading control normalisation. At least two replicates were performed for each experiment.

3.2.9.2 Relative quantification of TPO mRNA expression level using real time PCR

The Western blot results of the TPO expression were further validated by real time PCR method.

3.2.9.2.1 Elimination of false positive results in RT-PCR

TcRNA samples extracted from thyroid tissues with (RT-positive) and without (RT-negative) reverse transcription were amplified by PCR to confirm the total removal of genomic DNA from the sample after DNase I treatment. Primer pairs for the *Tata box-binding protein* (*TBP*) gene and *TPO* gene (E4F/ E5R) as listed in Table 3.12 were used in the PCR amplification. The PCR steps were performed as described in Section 3.2.5.1 except that cDNA was used as a template instead of genomic DNA. Positive control (genomic DNA) and negative control (RNA samples without reverse transcription) were also included in this study. Gel electrophoresis was carried out at 90 V for about 50 min. The separated bands were visualised and compared under UV light (300 nm wavelength) using a gel documentation system (Bio-Rad Version 2000).

3.2.9.2.2 Primer efficiency validation test

A standard curve for every primer set was plotted to determine its amplification efficiency of primer set in real time PCR analysis. The amplification reaction was carried out using 1 X SYBR® Green PCR mastermix (Applied Biosystems, USA), cDNA template with 2 fold or 10 fold serial dilution and 200 nM of each primer. The sample was initially denatured at 95 ℃ for 5 min followed by 40 cycles of subsequent denaturation at 95 ℃ for 15 sec and annealing elongation step at 60 ℃ for 1 min in a StepOnePlus system (Perkin-Elmer/Applied Biosystems, USA). The slope of the standard curve (Ct versus original mixture concentration, where C = product concentration and t = contact time) for every primer set should be as close to -3.321928 as possible to ensure high amplification efficiency.

Table 3.12 Sequence of PCR primers and size of PCR products in Section 3.2.9.2.1 and Section 3.2.9.2.3. Exon sequences are in uppercase; intron sequences are in lower case letters.

Genes	Exon (E) Intron (I)	Primers Sequence	Fragment size (bp)	Annealing temperature (℃)	Source
TBP	E4 E5	Forward 5'-CACGAACCACGGCACTGATT-3' Reverse 5'-TTTTCTTGCTGCCAGTCTGGAC-3'	89	59	Cristofaro *et al*., 2006
T*PO* (All known TPO transcripts)	E4 E5	Forward 5'-TCCAAACTTCCTGAGCCAAC-3' (E4F) Reverse 5'-CTCCTGTGATGGGCCTGTAT-3' (E5R)	241	59	This study
TPO (All known normally spliced transcript excluding TPO6	E12 E13	Forward 5'-TTCCTCAAGACGACAAGTGTG-3' (E12/E13F) Reverse 5'-CTGGAAATCCCATCCTTCCT-3' (E13R)	164	59	This study
TPO (Alternatively spliced derived transcripts)	E12/I12 E13 (c.2203 to c.2215…c.2216-34 to c.2370)	Forward 5'-ACCTTTCCTCAAGtttgactac-3' (E12/I12F) Reverse 5'-CTGGAAATCCCATCCTTCCT-3' (E13R)	202	59	This study
TPO (TPO6)	E11 E11/15	Forward 5'- GCTGGGAGGCTTAGTTGAAA-3' Reverse 5'- CCCGGAGTACCAGTCACCAT-3'	112	59	Cristofaro *et al*., 2006

3.2.9.2.3 Quantitative real time-polymerase chain reaction (qRT-PCR)

qRT-PCR method was used to quantify TPO expression in thyroid tissues of CHP33 (III-2) and her sister (III-1). The expression of TPO in the lesion area of thyroid tissue was normalised to that of the Tata box-binding protein (TBP) and compared with the expression of the same gene from normal area of thyroid tissue of same patient. Apart from this, the expression of TPO mRNA in CHP33 was also compared to her sister. Lastly, the ratio of TPO mRNA variants to total TPO mRNA was determined in each thyroid tissue sample. The total TPO mRNA expression was analysed using *TPO* primers amplifying a region from exons 4 to 5 (E4F and E5R primer pair). Primers pairs spanning from exons 12 to 13 were designed for separate quantification of the TPO variants. The E12/13F and E13R primer pair was used to target normally spliced transcripts except TPO6. Meanwhile, the E12/I12F and E13R primer pair was used to target alternatively spliced-derived transcripts with insertion of 34 bp between exon 12 and exon 13, which were reported in Section 4.3.1.2. Another pair of primer, E11 and E11/15, was used to amplify TPO6 that does not contain exon 12 and exon 13. The details of the primers used are shown in Table 3.12. qRT-PCR experiments were performed on a StepOnePlus system (Perkin-Elmer/Applied Biosystems). The amplification reaction was carried out using 1 X SYBR® Green PCR Mastermix (Applied Biosystems, USA), 10 ng of cDNA and 200 nM of each primer. The sample was initially denatured at 95 ℃ for 5 min followed by 40 cycles of subsequent denaturation at 95 ℃ for 15 sec and annealing elongation step at 60 ℃ for 1 min. Melting curves were performed to check the specificity of the amplicon. Each experiment was carried out in triplicate. Comparison of total or target gene mRNA expression was done by calculating the differences in the threshold cycles.

The fold change in expression between tissue samples was calculated by:

Fold Change = $2^{-\Delta\Delta C_t}$,

Where,

$\Delta\Delta C_t$ = ($Ct_{\text{target gene mRNA}}$ - Ct_{TBP}) lesion area - ($Ct_{\text{target gene mRNA}}$ - Ct_{TBP}) normal area

The fold change in expression between patients was calculated by:

Fold Change = $2^{-\Delta\Delta C_t}$,

Where,

$\Delta\Delta C_t$ = ($Ct_{\text{target gene mRNA}}$ - Ct_{TBP}) CHP33 - ($Ct_{\text{target gene mRNA}}$ - Ct_{TBP}) CHP33's sister

Total 10 ng of cDNA sample from normal and lesion areas thyroid tissue (5 ng template from each area) of each patient was used as the template.

Meanwhile, the ratio of TPO mRNA variants to total TPO mRNA in each tissue types was determined by the calculation below:

For normal area of thyroid tissue,

Targeted TPO mRNA species $_{\text{normal area}}$ / total TPO mRNA $_{\text{normal area}}$ = $2^{-\Delta C_t}$

For lesion area of thyroid tissue,

Targeted TPO mRNA species $_{\text{lesion area}}$ / total TPO mRNA $_{\text{lesion area}}$ = $2^{-\Delta C_t}$

3.2.10 Gene expression analysis of other thyroid hormone-related genes

The expression of *Tg*, *TSH-R* and *NIS* genes in both normal and lesion areas of thyroid tissue of CHP33 and her sister (III-1) was measured as described in Section 3.2.9.2.3. The expression of target genes was normalised to the expression of the *TBP* gene. The details of the primers used are shown in Table 3.13. Two replicates were performed in every experiment.

Table 3.13 Sequence of the primers used in real time PCR amplification and size of PCR products as described by Cristofaro *et al*. (2006) and Cianfarani *et al.* (2010).

Genes	Exon	Primers Sequence	Fragment size (bp)	Annealing temperature (℃)	Source
TBP	4 5	Forward 5'-CACGAACCACGGCACTGATT-3' Reverse 5'-TTTTCTTGCTGCCAGTCTGGAC-3'	89	59	Cristofaro *et al*., 2006
Tg	31-32 34-35	Forward 5'-CCGGAAGAAAGTTATACTGGAAG-3' Reverse5'-TTTGAGCAATGGGCTTCTG-3'	356	59	Cianfarani *et al, 2010*
TSH-R	4-5 7-8	Forward 5'-CCTCCTAAAGTTCCTTGGCATT-3' Reverse 5'-AGGTAAACAGCATCCAGCTTTG-3'	243	59	Cianfarani *et al, 2010*
NIS	1-2 3-4	Forward 5'-CACCAGCACCTACGAGTACC-3' Reverse 5'-CCCGGTCACTTGGTTCAG-3'	143	59	Cianfarani *et al, 2010*