

## ABSTRACT

Congenital hypothyroidism (CH) is a public health concern affecting 1 / 3000 - 4000 newborn babies. In reference to this, *thyroid peroxidase (TPO)* abnormality, typically inherited as autosomal recessive traits was found to be one of the causes of dys-hormonogenetic CH. Our group had previously identified a homozygous c.1159G>A mutation in exon 8 of the *TPO* gene of CHP41. In this study, the *TPO* gene of CHP41's family members was screened for the c.1159G>A mutation and the results showed that all family members carried the same mutation either in homozygous or heterozygous forms. In addition, another 20 unrelated cases of dys-hormonogenetic CH were also included in this study. DNA sequence analysis of the *TPO* gene in these 20 unrelated patients revealed the presence of five *TPO* mutations: three were novel (c.670\_672del in exon 7, c.1186C>T in exon 8 and c.1502T>G in exon 9) while another two had been previously reported (c.2268dup in exon 13 and c.2647C>T in exon 16). Moreover, 12 polymorphisms including two that are novel (c.1-192C>A in a GC box and c.180-6C>A at 6 bp upstream of exon 4), were also found in the 21 unrelated patients. This study shows that only individuals associated with either homozygous or compound heterozygous form of *TPO* mutation were affected with dys-hormonogenetic CH whereas family members of patients with one mutant allele remained asymptomatic. *In silico* functional analyses indicated that all of the six mutations affected normal activity of TPO protein. Furthermore, the novel c.180-6C>A polymorphism is predicted to reduce the intrinsic strength of the natural splice site of exon 4 which could lead to an activation of other potential splice sites. Meanwhile, it is also believed that the novel c.1-192C>A polymorphism in the GC box might alter the expression levels of *TPO* gene in an individual. Further investigation on patients with c.2268dup mutation through biochemical and gene expression analyses confirmed the devastating effects of

the mutation. A novel TPO mRNA transcript which was believed to be associated with nonsense-associated altered splicing (NAS) mechanism was detected in patients associated with the c.2268dup mutation. In addition, lower expression of TPO protein was also detected in thyroid tissues with lesions compared to those of normal areas in the same patients with c.2268dup. In conclusion, mutations in the *TPO* gene are an underlying genetic cause of CH with dyshormonogenesis in the current cohort of patients.

## ABSTRAK

Masalah hipotiroidisme kongenital (CH) merupakan penyakit kesihatan global yang menjejaskan kesihatan bayi yang baru lahir pada kadar 1 / 3000 - 4000. Ketidaknormalan gen *thyroid peroxidase (TPO)* yang diwarisi secara resesif autosomal telah didapati sebagai salah satu punca masalah CH yang diakibatkan oleh kelenjar tiroid yang tidak berfungsi atau berfungsi sebahagian sahaja. Kajian terdahulu yang telah kami jalankan telah mengenal pasti sejenis mutasi pada ekson 8 di gen *TPO* yang dikenali sebagai c.1159G>A pada pesakit CHP41. Dalam kajian ini, penyaringan mutasi gen yang sama telah dijalankan terhadap ahli keluarga CHP41 dan hasil kajian menunjukkan bahawa semua ahli keluarga membawa mutasi yang sama, sama ada dalam bentuk homozigus atau heterozigus. Di samping itu, kajian ini juga meneruskan usaha untuk mengenal pasti mutasi-mutasi gen *TPO* yang menyebabkan masalah CH di kalangan pesakit-pesakit lain yang mempunyai kelenjar tiroid. Analisis terhadap turutan DNA di gen *TPO* daripada 20 orang pesakit yang berasingan menunjukkan kewujudan lima jenis mutasi, di mana tiga jenis mutasi (c.670\_672del di ekson 7, c.1186C>T di ekson 8 and c.1502T>G di ekson 9) adalah penemuan terbaru (novel) manakala dua jenis mutasi lagi (c.2268dup di ekson 13 and c.2647C>T di ekson 16) telah dilaporkan. Selain itu, 12 polimorfisme yang lain termasuk dua polimorfisme novel (c.1-192C>A di kotak GC dan c.180-6C>A yang terletak di tempat 6 bp sebelum ekson 4) juga ditemui dalam kajian ini. Kajian ini menunjukkan bahawa hanya individu yang dikaitkan dengan mutasi *TPO* dalam bentuk homozigus atau heterozigus ganda (compound heterozygous) mempunyai masalah CH manakala ahli keluarga mereka yang membawa satu alel mutan kekal asimptomatik. Analisis *in silico* menunjukkan bahawa semua enam mutasi menjejaskan aktiviti mRNA ataupun protein TPO. Tambahan pula, penemuan polimorfisme c.180-6C>A diramalkan akan menurunkan kadar kekuatan

intrinsik bagi tapak pemotongan (splice site) yang semulajadi pada ekson 4 dan akan mengakibatkan pengaktifan tapak pemotongan lain yang lebih berpotensi. Seterusnya, polimorfisme c.1-192C>A yang terletak di kotak GC juga dipercayai akan mempengaruhi tahap ekspresi gen *TPO*. Kajian selanjutnya terhadap mutasi c.2268dup melalui analisis biokimia dan ekspresi gen telah membuktikan kesan buruk daripada mutasi tersebut dan mendedahkan sesuatu spesies mRNA *TPO* novel yang dipercayai dikaitkan dengan mekanisme NAS. Selain itu, analisis terhadap kadar ekspresi protein *TPO* daripada pesakit-pesakit yang mempunyai mutasi c.2268dup menunjukkan kadar ekspresi yang lebih rendah di kawasan tisu yang tidak normal berbanding dengan tisu yang diambil dari kawasan yang normal. Kesimpulannya, kajian ini menunjukkan bahawa mutasi dalam gen *TPO* merupakan sesuatu punca masalah CH di kalangan pesakit yang mempunyai kelenjar tiroid.

## ACKNOWLEDGEMENTS

My deep gratitude goes first to Assoc. Prof. Dr. Sarni Mat Junit, who had expectedly guided me and gave the advice, support and tremendously encouragements throughout my postgraduate education. Dr. Sarni's mentoring and encouragements have been especially valuable, and her early insights contributed to the success of this project.

Heartfelt thanks to Prof. Dr. Fatimah Harun from the Department of Paediatrics, University of Malaya Medical Centre for providing the patient's blood samples and clinical consultation. Her unwavering enthusiasm for congenital hypothyroidism study kept me constantly engaged with my research, and her personal generosity helped made my time in University of Malaya. I would also like to thank Dr. Muhammad Yazid Bin Jalaludin from Department of Paediatrics for his assistance in collecting the blood samples and with the clinical data. His kindness and an inspirational style also help in sustaining a positive atmosphere in which to do research.

I am very grateful to all members of the Molecular Biology Laboratory and Lipid and Nutrition Laboratory especially Yasmin, Ursula, Christina, Chor Yin, Nani, Kong and other members from the Department of Molecular Medicine for their help and time. Also, I would like to thank Dr. Rozana and Choon Han from the Department of Pharmacy for their superb technical assistance in designing three dimensional model of TPO protein. I would also like to express my gratitude to the university of Malaya for granting me with the UM Fellowship Award.

Last but not least, I wish to extend my heartfelt gratitude to my parents for their love, patience and unconditional supports. Without them, I would not have gone this far in life.

<b>TABLE OF CONTENTS</b>	<b>PAGE</b>
<b>ORIGINAL LITERARY WORK DECLARATION</b>	ii
<b>ABSTRACT</b>	iii
<b>ACKNOWLEDGEMENTS</b>	vii
<b>CONTENTS</b>	ix
<b>LIST OF FIGURES</b>	xviii
<b>LIST OF TABLES</b>	xxvi
<b>ABBREVIATIONS</b>	xxx
<b>LIST OF APPENDICES</b>	xxxvi
<b>CHAPTER 1: INTRODUCTION</b>	
1.1 Background	2
1.2 Objectives of the study	4
<b>CHAPTER 2: LITERATURE REVIEW</b>	
2.1 Thyroid gland	6
2.2 Thyroid hormones	6
2.3 Hypothyroidism	7
2.4 Neonatal transient hypothyroidism	9
2.5 Permanent congenital hypothyroidism	11
2.6 Biochemical screening and clinical diagnosis of CH	11
2.7 Treatment of CH	12
2.8 Thyroid dysmorphogenesis	13
2.9 Iodide organification defects	14
2.10 Goitre	14

2.11	Thyroid nodules	15
2.12	Follicular adenoma	16
2.13	Thyroid peroxidase ( <i>TPO</i> ) gene	17
2.14	Thyroid peroxidase enzyme	19
2.15	<i>TPO</i> gene mutations and polymorphisms	21
2.16	Expression of <i>TPO</i> , <i>TG</i> , <i>TSH-R</i> and <i>NIS</i> in thyroid nodules	25

### **CHAPTER 3: MATERIALS AND METHODS**

3.1	Materials	28
3.1.1	Chemicals and reagents	28
3.1.2	Kits	31
3.1.3	Primers	32
3.1.4	Antibodies	32
3.1.5	Apparatus and instruments	32
3.2	Methods	35
3.2.1	Study design	35
3.2.2	Subjects for the <i>TPO</i> mutational screening	36
3.2.2.1	CHP41 and his family members	40
3.2.2.2	CHP33 and her family members	40
3.2.2.3	CHP49 and her family members	42
3.2.2.4	CHP51 and his family members	45
3.2.2.5	CHP53 and her family members	45
3.2.2.6	CHP55 and her family members	47
3.2.2.7	CHP58 and his family members	50
3.2.2.8	CHP59 and his family members	50
3.2.3	Blood sampling and genomic DNA extraction	53

3.2.4	Determination of the yield and quality of the extracted genomic DNA	54
3.2.5	Mutational analysis of the <i>TPO</i> gene	54
3.2.5.1	Polymerase chain reaction (PCR)	55
3.2.5.2	Agarose gel electrophoresis	60
3.2.5.3	Purification of the PCR product	61
3.2.5.3.1	Purification of the PCR product by QIAquick® PCR Purification Kit (Qiagen, Germany)	61
3.2.5.3.2	Purification of the PCR product by MinElute® Gel Extraction Kit (Qiagen, Germany)	62
3.2.5.4	Analysis of the purified PCR product	63
3.2.5.5	DNA sequencing of the PCR product	63
3.2.6	Designing new primer pairs	63
3.2.7	Assessing the significance of nucleotide sequence alterations in the <i>TPO</i> gene on protein functions using computational methods	64
3.2.7.1	Assessing the potential impact of <i>TPO</i> sequence alterations on splicing activity using HSF algorithm	64
3.2.7.2	Multiple amino acid sequence alignment	65
3.2.7.3	SIFT and PolyPhen-2	65
3.2.7.4	PSIPRED	65
3.2.7.5	Tertiary structure prediction	66

3.2.7.6	The search for transcription factors: TATA box, CAAT box, GC box in upstream region of the <i>TPO</i> gene	66
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	67
3.2.8.1	Total cellular RNA isolation	67
3.2.8.2	Elimination of genomic DNA from RNA samples by DNase I treatment	68
3.2.8.3	Determination of the yield, quality and integrity of tcRNA	69
3.2.8.4	Reverse transcription of tcRNA to cDNA	69
3.2.8.5	Confirmation of the <i>in silico</i> HSF analysis result of the mutation c2268dup mutation	70
3.2.8.6	Genetic analysis of the upstream region of exon 13 of the <i>TPO</i> gene in CHP33	70
3.2.8.7	Microsomal proteins isolation	72
3.2.8.8	Bradford protein assay	72
3.2.8.9	SDS-PAGE and Western blot	73
3.2.8.9.1	Gel apparatus assembly for SDS-PAGE	73
3.2.8.9.2	Preparation of polyacrylamide gel (PAGE)	73
3.2.8.9.3	Sample preparation for SDS-PAGE	74
3.2.8.9.4	Electrophoresis	75

3.2.8.9.5 Staining of proteins in gels with Coomassie stain	75
3.2.8.9.6 Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets	76
3.2.8.9.7 Immunodetection	76
3.2.8.10 Guaiacol oxidation assay	77
3.2.9 Quantification for expression of TPO in thyroid tissue of CHP33 (III-2) and her sister (III-1)	78
3.2.9.1 Relative quantification of TPO proteins on Western Blots	78
3.2.9.2 Relative quantification of TPO mRNA expression level on real time PCR	78
3.2.9.2.1 Elimination of false positive results in RT-PCR	79
3.2.9.2.2 Primer efficiency validation test	79
3.2.9.2.3 Quantitative real time polymerase chain reaction (qrt-PCR)	81
3.2.10 Gene expression analysis of other thyroid hormone-related genes	83

## **CHAPTER 4: RESULTS**

4.1 Screening for <i>TPO</i> gene alterations in unrelated patients with dysmorphogenetic CH and their family members	86
4.1.1 DNA isolation and quantification	86
4.1.2 PCR optimisation	87

4.1.3	Detection of <i>TPO</i> gene mutations in unrelated patients with dys hormonogenetic CH	87
4.1.4	Detection of <i>TPO</i> gene polymorphisms in unrelated patients with dys hormonogenetic CH	98
4.1.5	Detection of <i>TPO</i> gene mutation in family members of CHP41, CHP33, CHP53, CHP58 and CHP59	133
4.1.5.1	Detection of the c.1159G>A (p.Gly387Arg) mutation in family members of CHP41 (index patient, II-4)	133
4.1.5.2	Detection of the c.2268dup (p.Glu757X) mutation analysis in family members of CHP33 (index patient, III-2)	138
4.1.5.3	Detection of the c.1502T>G (p.Val501Gly) mutation in family members of CHP53 (index patient, II-1)	138
4.1.5.4	Detection of the c.670_672del (p.Asp224del) and c.2268dup (p.Glu757X) mutations in family members of CHP58 (index patient, II-1)	145
4.1.5.5	Detection of the c.2268dup (p.Glu757X) mutation in family members of CHP59 (index patient, III-2)	145
4.2	<i>In silico</i> analysis to predict the functional impact of the nucleotide sequence alteration(s) in the <i>TPO</i> gene	152

4.2.1	Analysis of the c.670_672del (p.Asp224del) mutation	152
4.2.2	Analysis of the c.1159G>A (p.Gly387Arg) mutation	156
4.2.3	Analysis of the c.1186C>T (p.Arg396Cys) mutation	173
4.2.4	Analysis of the c.1502T>G (p.Val501Gly) mutation	189
4.2.5	Analysis of the c.2268dup (p.Glu757X) mutation	197
4.2.6	Analysis of the c.2647C>T (p.Pro883Ser) mutation	205
4.2.7	Analysis of the novel c.1-982C>A (GC box-like region, GCGCGG>GCGCAG) polymorphism	218
4.2.8	Analysis of the novel c.180-6C>A polymorphism	218
4.3	The effects of the c.2268dup (p.Glu757X) mutation in exon 13 on the TPO pre-mRNA splicing activity, protein enzymatic activity, the expression of <i>TPO</i> gene/protein and other thyroid hormone-related genes	223
4.3.1	Analysis of TPO transcripts in thyroid tissues of patients with c.2268dup (p.Glu757X) mutation	223
4.3.1.1	Isolation and assessment of the integrity of tcRNA	223
4.3.1.2	Detection and identification of a novel alternative splicing-derived TPO mRNA variant in CHP33 (index patient, III-2) and her sister (III-1) associated with the c.2268dup (p.Glu757X) mutation	225
4.3.2	Biochemical analysis of the p.Glu757X mutant	239
4.3.2.1	Protein assay	239

4.3.2.2 SDS-PAGE and Western blot	239
4.3.2.3 Peroxidase activity of p.Glu757X mutant	242
4.3.3 Quantification of TPO mRNA and protein in CHP33 (III-2) and her sister (III-1)	242
4.3.3.1 Western Blot	242
4.3.3.2 Quantification of TPO and other thyroid hormone-related genes	245
4.3.3.2.1 <i>TPO</i> gene expression differences between the normal and lesion areas of thyroid tissue of CHP33 (III-2) and her sister (III-1)	248
4.3.3.2.2 The <i>TPO</i> gene expression differences between CHP33's sister (III-1) and CHP33 (III-2)	249
4.3.3.2.3 Quantification of TPO mRNA variants	249
4.3.3.2.4 <i>Tg</i> , <i>TSH-R</i> , <i>NIS</i> genes expression difference between normal and lesion areas of thyroid tissue of CHP33 (III-2) and her sister (III-1)	254

## **CHAPTER 5: DISCUSSION**

5.0 Discussion	261
----------------	-----

**CHAPTER 6: CONCLUSION, LIMITATIONS AND  
SUGGESTIONS FOR FUTURE RESEARCH**

6.1	Conclusion	279
6.2	Limitations and suggestions for future research	280
	<b>REFERENCES</b>	282

<b>LIST OF FIGURES</b>	<b>PAGE</b>
2.1 Schematic diagram of a follicular cell, illustrating the steps involved in thyroid hormone synthesis	8
2.2 Human TPO mRNA transcripts	18
2.3 Schematic diagram of the structure of human TPO	20
2.4 A schematic diagram showing inactivating mutations reported in the coding region of the human <i>TPO</i> gene	22
4.1 An agarose gel electrophoresis of PCR products of exons 1 to 6 of the <i>TPO</i> gene after purification	88
4.2 An agarose gel electrophoresis of PCR products of exons 7 to 12 of the <i>TPO</i> gene after purification	89
4.3 An agarose gel electrophoresis of PCR products of exons 13 to 17 of the <i>TPO</i> gene after purification	90
4.4 Electropherograms showing a c.670_672del mutation in exon 7 of the <i>TPO</i> gene	92
4.5 Electropherograms showing a c.1186C>T mutation in exon 8 of the <i>TPO</i> gene	93
4.6 Electropherograms showing a c.1502T>G mutation in exon 9 of the <i>TPO</i> gene	94
4.7 Electropherograms showing a c.2268dup mutation in exon 13 of the <i>TPO</i> gene	95
4.8 Electropherograms showing a c.2647C>T mutation in exon 16 of the <i>TPO</i> gene	97
4.9 Electropherograms showing a c.1-982G>A polymorphism in GC box-like region (GCGCGG) of the <i>TPO</i> gene	99

4.10	Electropherograms showing a c.180-6C>A polymorphism at 6 bp upstream of exon 4 of the <i>TPO</i> gene	101
4.11	The frequency of alleles G (c.1-982G) and A (c.1-982A) of the <i>TPO</i> gene in 30 normal individuals	102
4.12	The frequency of alleles C (c.180-6C) and A (c.180-6A) of the <i>TPO</i> gene in 30 normal individuals	103
4.13	Electropherograms showing a c.1-937A>G polymorphism in 5'UTR region of the <i>TPO</i> gene	104
4.14	Electropherograms showing a c.12C>G polymorphism in exon 2 of the <i>TPO</i> gene	108
4.15	Electropherograms showing a c.769G>T polymorphism in exon 7 of the <i>TPO</i> gene	110
4.16	Electropherograms showing a c.1117G>T polymorphism in exon 8 of the <i>TPO</i> gene	112
4.17	Electropherograms showing a c.1193G>C polymorphism in exon 8 of the <i>TPO</i> gene	116
4.18	Electropherograms showing a c.1728G>A polymorphism in exon 10 of the <i>TPO</i> gene	120
4.19	Electropherograms showing a c.1998C>T polymorphism in exon 11 of the <i>TPO</i> gene	121
4.20	Electropherograms showing a c.2145C>T polymorphism in exon 12 of the <i>TPO</i> gene	123
4.21	Electropherograms showing a c.2173A>C polymorphism in exon 12 of the <i>TPO</i> gene	125
4.22	Electropherograms showing a c.2540T>C polymorphism in exon 15 of the <i>TPO</i> gene	127

4.23	A summary of <i>TPO</i> gene alterations found in all unrelated patients with dys hormonogenetic CH in this study	130
4.24	Electropherograms showing a <i>TPO</i> gene c.1159G>A mutation in CHP41 and his family members	134
4.25	A family pedigree of CHP41 demonstrates the inheritance mode of the c.1159G>C mutation	137
4.26	Electropherograms showing a <i>TPO</i> gene c.2268dup mutation in family members of CHP33	139
4.27	A family pedigree of CHP33 demonstrates the inheritance mode of the c.2268dup mutation	141
4.28	Electropherograms showing a <i>TPO</i> gene c.1502T>G mutation in family members of CHP53	142
4.29	A family pedigree of CHP53 demonstrates the inheritance mode of the c.1502G>T mutation	144
4.30	Electropherograms showing a <i>TPO</i> gene c.670_672del mutation in CHP58's father (I-1)	146
4.31	Electropherograms showing a <i>TPO</i> gene c.2268dup mutation in CHP58's mother (I-2)	147
4.32	A family pedigree of CHP58 demonstrates the inheritance mode of the c.670_672del and c.2268dup mutations	148
4.33	Electropherograms showing a <i>TPO</i> gene c.2268dup mutation in family members of CHP59	149
4.34	A family pedigree of CHP59 demonstrates the inheritance mode of the c.2268dup mutation	151
4.35	Homology model of the wild-type human TPO using sheep lactoperoxidase as a template (PDB_2IKC)	153

4.36	Multiple sequence alignment of amino acids in human TPO with different animal species (p.Asp224del)	157
4.37	The predicted secondary structures of amino acid sequence of the (a) wild type and the (b) mutant (p.Asp224del)	158
4.38	Computer generated models illustrating the comparison between wild type and mutant p.Asp224del TPO proteins	159
4.39	Multiple sequence alignment of amino acids in human TPO with different animal species (p.Gly387Arg)	169
4.40	Analysis of p.Gly387Arg nucleotide transition by PolyPhen-2	170
4.41	Analysis of p.Gly387Arg nucleotide transition using SIFT	171
4.42	The predicted secondary structures of amino acid sequence of the (a) wild type and the (b) mutant (p.Gly387Arg)	172
4.43	Computer generated models illustrating the comparison between wild type and mutant p.Gly387Arg TPO proteins	174
4.44	Multiple sequence alignment of amino acids in human TPO with different animal species (p.Arg396Cys)	181
4.45	Analysis of p.Arg396Cys nucleotide transition by PolyPhen-2	182
4.46	Analysis of p.Arg396Cys nucleotide transition using SIFT	183
4.47	The predicted secondary structures of amino acid sequence of the (a) wild type and the (b) mutant (p.Arg396Cys)	184
4.48	Computer generated models illustrating the comparison between wild type and mutant p.Arg396Cys TPO proteins	185
4.49	Multiple sequence alignment of amino acids in human TPO with different animal species (p.Val501Gly)	194

4.50	Analysis of p.Val501Gly nucleotide transversion by PolyPhen-2	195
4.51	Analysis of p.Val501Gly nucleotide transversion using SIFT	196
4.52	The predicted secondary structures of amino acid sequence of the (a) wild type and the (b) mutant (p.Val501Gly)	198
4.53	Computer generated models illustrating the comparison between wild type and mutant p.Val501Gly TPO proteins	199
4.54	A schematic diagram showing the comparison between the 3-D models of wild type and c.2268dup (p.Glu757X) mutant proteins	208
4.55	Multiple sequence alignment of amino acids in human TPO with different animal species (p.Pro883Ser)	213
4.56	Analysis of p.Pro883Ser nucleotide transition by PolyPhen-2	214
4.57	Analysis of p.Pro883Ser nucleotide transition by SIFT	215
4.58	A schematic diagram showing the location of the TPO transmembrane (Ser-853 to Thr-868) and intracellular (Val-869 to Leu-933) domains	216
4.59	The predicted secondary structures of amino acid sequence of the (a) wild type and the (b) mutant (p.Pro883Ser)	217
4.60	Comparison between the 3-D homology models of wild type and mutant p.Pro883Ser TPO proteins	219
4.61	A schematic diagram presentation of the potential GC box binding site in upstream region (-200 to -1 bp) of transcription start of the <i>TPO</i> gene, identified by TFBIND	220

4.62	Gel electrophoresis of DNase I-treated tcRNA samples showing two discrete rRNA bands representing the 28S rRNA and 18S rRNA	224
4.63a	An agarose gel electrophoresis showing the PCR products of exons 2 to 13 that include the pre-mRNA splice junctions	226
4.63b	An agarose gel electrophoresis showing the PCR products of exons 7 to 9 and exons 13 to 17 that include the pre-mRNA splice junctions	227
4.64a	An agarose gel electrophoresis showing the PCR products of exons 12 to 13 that include the pre-mRNA splice junction	228
4.64b	An agarose gel electrophoresis showing the PCR products of exons 12 to 13 that include the pre-mRNA splice junction	229
4.65a	Electropherogram profile of the expected PCR product with the size of 300 bp obtained from the amplification spanning from TPO exon 12 and end at exon 13 of CHP33	230
4.65b	Electropherogram profile of the unknown PCR product with the size of approximately 350 bp showing the detection of a novel alternative splicing-derived TPO mRNA variant with additional length of 34 bp originated from intron 12 in CHP33	231
4.66	An agarose gel electrophoresis showing the PCR product for wild type and unknown TPO transcripts	232

4.67	An electropherogram showing identification of a novel alternative splicing-derived TPO mRNA variant	233
4.68	A schematic diagram showing the consequences of the c.2268dup mutation on the production of TPO mRNA transcripts and mediated the subsequent synthesis of the TPO polypeptide	235
4.69	Analysis of p.Asp740Valfs* amino acid substitution by PolyPhen-2	237
4.70	Electropherogram profile of the PCR product showing c.2216-112 at intron 12 to exon 13 of the <i>TPO</i> gene of CHP33	238
4.71	A SDS-PAGE of microsomal fraction extracts and a positive control	240
4.72	Western blot analysis was performed with protein extracted from microsomal fraction of thyroid tissues	241
4.73	Guaiacol oxidation assay of p.Glu757X TPO mutant	243
4.74	Western blot analysis: Comparison of <i>TPO</i> protein expression in CHP33 (III-2) and her sister (III-1)	244
4.75	An agarose gel electrophoresis of PCR products of RT-PCR sensitivity test	246
4.76	Real time PCR analysis: Comparison of the <i>TPO</i> gene expression level between the normal and lesion areas of thyroid tissue of CHP33 (III-1)	250

4.77	Real time PCR analysis: Comparison of the <i>TPO</i> gene expression level between the normal and lesion areas of thyroid tissue of CHP33's sister (III-1)	251
4.78	Real time PCR analysis: Comparison of the <i>TPO</i> gene expression between CHP33 (III-2) and her sister (III-1)	252
4.79	Real time PCR analysis: The sum (%) of examined <i>TPO</i> variants in normal and lesion areas of thyroid tissue of CHP33 (III-2)	255
4.80	Real time PCR analysis: The sum (%) of examined <i>TPO</i> variants in normal and lesion areas of thyroid tissue of CHP33's sister (III-1)	256
4.81	Real time PCR analysis: Comparison of <i>TG</i> , <i>TSH-R</i> and <i>NIS</i> genes expression between normal and lesion areas of thyroid tissue of CHP33 (III-2)	258
4.82	Real time PCR analysis: Comparison of <i>TG</i> , <i>TSH-R</i> and <i>NIS</i> genes expression between normal and lesion areas of thyroid tissue of CHP33's sister (III-1)	259

<b>LIST OF TABLES</b>	<b>PAGE</b>
2.1 Clinical features of hypothyroidism	10
2.2 A summary of published <i>TPO</i> polymorphisms	23
3.1 Profile of patients with dys hormonogenetic CH showing the respective thyroid function and status at the time of diagnosis	37
3.2 Clinical profile of family members of CHP41 with dys hormonogenetic CH	41
3.3 Clinical profile of family members of CHP33 with dys hormonogenetic CH	43
3.4 Clinical profile of family members of CHP49 with dys hormonogenetic CH	44
3.5 Clinical profile of family members of CHP51 with dys hormonogenetic CH	46
3.6 Profile of family members of CHP53 with dys hormonogenetic CH	48
3.7 Profile of family members of CHP55 with dys hormonogenetic CH	49
3.8 Profile of family members of CHP58 with dys hormonogenetic CH	51
3.9 Profile of family members of CHP59 with dys hormonogenetic CH	52
3.10 Sequence of primers used for PCR amplification of exons 1 to 17 of the <i>TPO</i> gene, size of PCR products and the annealing temperature needed for each pair of primers	56

3.11	Nucleotide sequence of PCR primers and the size of PCR products for mRNA transcript analysis of the <i>TPO</i> gene	71
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	80
3.13	Sequence of the primers used in real time PCR amplification and size of PCR products as described by Cristofaro <i>et al.</i> (2006) and Cianfarani <i>et al.</i> (2010)	84
4.1	A summary of the disease-causing <i>TPO</i> gene mutations found in all unrelated patients with dys hormonogenetic CH	131
4.2	A summary of the <i>TPO</i> gene polymorphisms found in 14 unrelated patients with dys hormonogenetic CH	132
4.3	The location and nucleotide sequence of splice sites found in exon 7 of the <i>TPO</i> gene	154
4.4	ESE Finder matrices for SRp40, SC35, SF2/ASF and SRp55 proteins (c.670_672del)	155
4.5	HSF ESE motif analysis for Tra2 and 9G8 proteins: Splicing enhancer motif of the c.670_672del mutation	155
4.6	The location and nucleotide sequence of splice sites found in exon 8 of the <i>TPO</i> gene	164
4.7	Splice site analysis of the c.1159G>A mutation using HSF	166
4.8	ESE Finder matrices for SRp40, SC35, SF2/ASF and SRp55 proteins (c.1159G>A)	167
4.9	HSF ESE motif analysis for Tra2 and 9G8 proteins: Splicing enhancer motif of the c.1159G>A mutation	168
4.10	Splice site analysis of the c.1186C>T mutation using HSF	178

4.11	ESE Finder matrices for SRp40, SC35, SF2/ASF and SRp55 proteins (c.1186C>T)	179
4.12	The location and nucleotide sequence of splice sites found in exon 9 of the <i>TPO</i> gene	190
4.13	Splice site analysis of the c.1502T>G mutation using HSF	191
4.14	ESE Finder matrices for SRp40, SC35, SF2/ASF and SRp55 proteins (c.1502T>G)	192
4.15	HSF ESE motif analysis for Tra2 and 9G8 proteins: Splicing enhancer motif of the c.1502T>G mutation	193
4.16	The location and nucleotide sequence of splice sites found in exon 13 of the <i>TPO</i> gene	203
4.17	Splice site analysis of the c.2268dup mutation using HSF	204
4.18	ESE Finder matrices for SRp40, SC35, SF2/ASF and SRp55 proteins (c.2268dup)	206
4.19	HSF ESE motif analysis for Tra2 and 9G8 proteins: Splicing enhancer motif of the c.2268dup mutation	207
4.20	The location and nucleotide sequence of splice sites found in exon 16 of the <i>TPO</i> gene	209
4.21	Splice site analysis of the c.2647C>T mutation using HSF	211
4.22	ESE Finder matrices for SRp40, SC35, SF2/ASF and SRp55 proteins (c.2647C>T)	212
4.23	The location and nucleotide sequence of splice sites found in exon 4 of the <i>TPO</i> gene	221
4.24	Splice site analysis of the c.180-6C>A (c.181-6C>A) polymorphism using HSF	222

4.25	Comparison between the primer pair amplification efficiency of the target genes: <i>thyroid peroxidase (TPO)</i> , <i>thyroglobulin (Tg)</i> , <i>thyroid stimulating hormone receptor (TSH-R)</i> , and <i>sodium iodide symporter (NIS)</i> , and endogenous control: <i>tata box binding protein (TBP)</i>	247
------	---	-----

## ABBREVIATIONS

A (a)	adenine
Ala	alanine
APS	ammonium persulfate
Arg	arginine
Asn	asparagine
Asp	aspartic acid
bp	base pair
BSA	bovine serum albumin
Bis	N, N'-methylene-bis-acrylamide
C (c)	cytosine
C (cell)	calcitonin-producing parafollicular
C (product)	concentration
C (terminal)	carboxyl-terminus
CH	congenital hypothyroidism
CHP	congenital hypothyroidism patient
CCP	complement control protein
cDNA	complementary DNA
cm	centimeter
CO <sub>2</sub>	carbon dioxide
ddH <sub>2</sub> O	double-distilled water
dl	deciliter
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphates

DTT	dithiothreitol
DUOX2	dual oxidase 2
EDTA	ethylenediaminetetraacetate
EGF	epidermal growth factor
ESE(s)	exonic splicing enhancer(s)
ESSs	exonic splicing suppressors
<i>et al.</i>	et alia (and others)
EtBr	ethidium bromide
FNAB	fine needle aspiration biopsy
FT <sub>4</sub>	free T <sub>4</sub>
G (g)	guanine
g	gram
g	gravity
Gln	glutamine
Glu	glutamic acid
Gly	glycine
h	hour(s)
H <sub>2</sub> O <sub>2</sub>	hydrogen peroxide
HCl	hydrochloride
His	histidine
HSF	Human Splicing Finder
hTG	human thyroglobulin
I <sup>-</sup>	iodide
I <sup>+</sup>	iodinium
ICH-GCP	International Conference on Harmonisation-Good Clinical Practice

Ile	isoleucine
IQ	intelligence quotient
K <sup>+</sup>	potassium ion
kbp	kilo base pair
KCl	potassium chloride
kDa	kiloDalton
K <sub>3</sub> EDTA	ethylenediamine tetraacetate
kg	kilogram
L	litre
Leu	leucine
LPO	lactoperoxidase
L-T <sub>4</sub>	levothyroxine
L-T <sub>3</sub>	liothyronine
Lys	lysine
m	mili
M	molar
Met	methionine
MeOH	Methanol
U (u)	uracil
μIU	microinternational units
μg	microgram
mg	milligram
MgCl <sub>2</sub>	magnesium chloride
min	minute(s)
ml	milliliter
μl	microlitre

mcg	microgram
mM	millimolar
MNG	multinodular goitre
MPO	myeloperoxidase
mRNA	messenger ribonucleic acid
N (terminal)	amino-terminus
Na <sup>+</sup>	sodium ions
NaCl	sodium chloride
NaOH	sodium hydroxide
NAS	nonsense-associated altered splicing
NCBI	National Center for Biotechnology Information
NIS	sodium iodide symporter
NTH	Neonatal transient hypothyroidism
nmol	nanomolar
OD <sub>230</sub>	absorbance at 230 nm
OD <sub>260</sub>	absorbance at 260 nm
OD <sub>280</sub>	absorbance at 280 nm
OD <sub>470</sub>	absorbance at 470 nm
OD <sub>595</sub>	absorbance at 595 nm
PAGE	polyacrylamide gel
PAX-8	paired box gene 8
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Phe	phenylalanine
PIOD	partial organification defect
pmol	picomole

Polyphen-2	Polymorphism Phenotyping-version 2
Pro	proline
ProQ	Protein Quality Predictor
PVDF	polyvinylidene fluoride
qRT-PCR	quantitative real time-polymerase chain reaction
RT-PCR	reverse transcription-polymerase chain reaction
SCN	solid cell nest
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sec	second(s)
Ser	serine
SIFT	Sorting Intolerant From Tolerant
SLC5A	solute carrier family 5
SNP	single nucleotide polymorphism
t	time
T (t)	thymine
T <sub>3</sub>	3, 5, 3'-triiodothyronine
T <sub>4</sub>	3, 5, 3', 5'-tetraiodothyronine or thyroxine
<i>Taq</i>	<i>Thermus aquaticus</i>
TAE	tris-acetate-EDTA
TBP	tata box binding protein
tcRNA	total cellular RNA
TDH2A	thyroid dyshormonogenesis 2A
TEMED	tetramethylethylenediamine
TFT	thyroid function test
Tg	thyroglobulin

Thr	threonine
TIOD	total iodide organification defect
TNH	transient neonatal hypothyroidism
TPO	thyroid peroxidase
TRH	thyroid releasing hormone
Trp	tryptophan
TSH	thyroid stimulating hormone
TSHB	thyroid stimulating hormone, beta
TSHR	thyroid stimulating hormone receptor
TTF-1	thyroid transcription factor-1
TTF-2	thyroid transcription factor-2
Tyr	tyrosine
UMMC	University of Malaya Medical Central
UniProt	Universal Protein Resource
UV	ultraviolet
V	volt
Val	valine
v/v	volume over volume
w/v	weight over volume
3-D	three dimensional
<sup>99</sup> Tm	Technetium-99m
°C	degree centigrade
Δ	Delta

<b>LIST OF APPENDICES</b>	<b>PAGE</b>
A List of publications in this study	299
B List of posters presented in this study	300
C The published sequence of the:	301
1) Human thyroid peroxidase (TPO), transcript variant 1, mRNA (NCBI Reference Sequence: NM_000547.5)	
2) Human thyroid peroxidase (TPO), isoform 1, protein (Uniprot Reference Sequence: P07202-1)	
3) Human thyroid peroxidase (TPO), RefSeqGene on chromosome 2 (NCBI Reference Sequence: NG_011581.1, selected region from 4799 to 5949)	
4) Human thyroid peroxidase (TPO), RefSeqGene on chromosome 2 (NCBI Reference Sequence: NG_011581.1, selected region from 24969 to 25146)	
5) Human thyroid peroxidase (TPO), RefSeqGene on chromosome 2 (NCBI Reference Sequence: NG_011581.1, selected region from 87993 to 88308)	
D Recipe for stock solutions and general use buffers	304
1) Preparation of 50 X Tris-acetate-EDTA (TAE) buffer	
2) Preparation of 6 X Laemmli buffer	
3) Preparation of 10 X SDS-PAGE running buffer	
4) Preparation of Coomassie stain	
5) Preparation of 1 X Tris-glycine buffer	
E Exon-exon boundary for all exons of the TPO variants	306

F	BSA standard curve	311
G	The expression level of TPO protein in:	312
	1) CHP33 (III-2)	
	2) CHP33's sister (III-1)	
	3) CHP33's sister (III-1) and CHP33 (III-2) (comparison)	
H	Multiple sequence alignment of amino acids in human TPO with rat TPO	317
I	Ethical committee approval letter	318
J	Patient consent form for clinical research	319