5.0 DISCUSSION

Permanent CH is one of the world's most common endocrine disorders affecting 1/3000 - 4000 newborn babies every year (Amar-Singh, 2010; Delange, 1979; Fisher *et al.*, 1979; Gruters & Krude, 2007; Rastogi & LaFranchi, 2010). In Malaysia, neonatal hypothyroid screening program has been conducted in many government, private and university hospitals since year 1998 but not nationally to allow early detection and treatment of CH, thus preventing the developmental delay, growth failure and other complications that result from the lack of thyroid hormone (Amar-Singh, 2010).

Thyroid scintigraphy and ultrasound scanning are usually carried out to determine the status of thyroid gland before treatment is instituted, or at the age of 3 years, when it is safe to put off the T_4 replacement therapy temporarily before scanning (Rose *et al.*, 2006). The gland can be classified as normal, aplastic, ectopic, hypoplastic glands or a goitre. In this study, all of the affected patients were confirmed to have hypothyroidism by biochemical evidence of high TSH and low FT4 and presence of normal thyroid gland on thyroid scan except for CHP49 who had not reached the age of 3 when this study was carried out. Some of our patients who were born in rural areas or before the neonatal screening program was introduced had suffered the consequences of late treatment, affecting their growth and cognitive function with low IQ range.

Our group had earlier screened for mutations in the thyroid-specific genes including *TSH-R*, *TTF-2*, *PAX-8* and *TPO* in Malaysian patients with CH (Kang, 2010; Musa *et al.*, 2006; Yong *et al.*, 2007). The studies revealed a total of two mutations and 16 polymorphisms (one mutation and four polymorphisms in the *TTF-2* gene; eight polymorphisms in the *TSHR* gene; two polymorphisms in the *PAX8* gene; one mutation

and two polymorphisms in the *TPO* gene). In this study, mutational screening of the *TPO* gene was continued in 21 unrelated patients with dyshormonogenetic CH. A total of five *TPO* mutations: c.670_672del, c.1186C>T, c.1502T>G, c.2268dup, c.2647C>T; and 12 *TPO* polymorphisms: c.1-982G>A, c.1-937A>G, c.12C>G, c.180-6C>A, c.769G>T, c.1117G>T, c.1193G>C, c.1728G>A, c.1998C>T, c.2145C>T, c.2173A>C and c.2540T>C were detected in these patients. Among the *TPO* alterations, the c.670_672del, c.1186C>T, c.1502T>G mutations and c.1-982G>A, c.180-6C>A polymorphisms have not been described in any public SNP databases.

Kang (2010) had previously identified a documented mutation, c.1159G>A in exon 8 of the *TPO* gene, in CHP41 (II-4) with dyshormonogenetic CH and his sister (II-3). CHP41 and II-3 were shown to carry the mutation in a homozygous form. The c.1159G>A nucleotide transition results in a change of glycine to arginine at codon 387 (p.Gly387Arg). Comparison of amino acid sequence of human TPO with other species revealed that glycine at codon 387 is not well conserved suggesting that the glycine at this position is probably not critical for the catalytic activity of the TPO (Kang, 2010). SIFT and Polyphen analyses carried out by Ris-Stalpers & Bikker in year 2010 also predicted that the variant is harmless and did not modify TPO function. The mutation is however, located within the heme-binding catalytic region of the TPO. The protein secondary prediction analysis carried out by Rivolta *et al.* in year 2007 had shown that the mutation caused structural changes to the TPO and thus postulated that this mutation would indirectly interfere with the TPO activity.

To further evaluate the effect of this mutation on the TPO enzymatic function, in this study, a homology 3-D model of the human TPO p.Gly387Arg mutant was generated for structural comparison. It was found that the structural alterations in the

mutant protein were distant from the important functional sites thus strengthen the hypothesis that this mutation does not directly affect enzymatic site of the TPO protein.

On the other hand, recent reports have shown that mutations in exonic sequences might disrupt the sequence recognised by splicing regulators such as exonic splicing enhancers (ESEs) or create exonic splicing suppressors (ESSs) (Sterne-Weiler *et al.*, 2011; Warf & Berglund, 2010). Therefore, interruption in these sequences can lead to abnormal splicing or exon skipping. In the present study, *in silico* analyses revealed that sequence alteration caused by the c.1159G>A mutation is predicted to create a new acceptor splice site at c.1150 for exon 8 of the *TPO* gene and possibly give rise to production of a TPO transcript 341 bp shorter than that of the wild type. Alternatively, the alteration in the ESE-binding site sequence due to the c.1159G>A mutation might interrupt the correct splicing of pre-mRNA and lead to the activation of other potential splice sites in exon 8 which subsequently give rise to the production of various TPO protein isoforms. Nonetheless, the whole of exon 8 maybe naturally spliced to produce a TPO mRNA variant (TPO5), where the translated product is not able to acquire a proper three dimensional configuration and unable to reach the cell surface (Ferrand *et al.*, 2003). Therefore, the alteration of the ESE-binding site sequence in the c.1159G>A mutant can also possibly influence the excision rate of exon 8 during the pre-mRNA splicing. Since exon 8 of the *TPO* gene has been suggested to code for a site that participates in catalytic mechanism, thus, any modifications or absence of this critical region can cause the translated TPO protein devoid of or reduced in, enzymatic activity.

In this study, c.1159G>A mutation was also detected in other family members of CHP41. DNA sequencing analysis revealed that the healthy CHP41's father, mother and one of the sisters were all heterozygotes for the c.1159G>A mutation. Their eldest

sister (II-1) has also been found to have the same c.1159G>A mutation but in homozygous form. However, II-1 appeared to be asymptomatic until the age of 19 where she started to develop goitre. She was later diagnosed biochemically with hypothyroidism without other clinical symptoms. Although the current study could not explain why II-1 and II-3 had only developed a goitre in later life despite harbouring the same mutation and polymorphisms (data not shown) in the *TPO* gene, we postulate that the CH in CHP41 could be due to mutations in other thyroid-specific genes such as *DUOX2* (Varela *et al.*, 2006), *NIS* (Pohlenz & Refetoff, 1999), *TG* (Ieiri *et al.*, 1991) or *TSHB* (Deladoey *et al.*, 2003) along with the c.1159G>A mutation in the *TPO* gene. Noteworthy, all subjects with homozygous c.1159G>A in this study had developed goitres. It is very likely that the goitres are associated with homozygous c.1159G>A mutation. A recent study has shown that the differential expression of TPO5 is associated with thyroid malignancy (Prasad *et al.*, 2008). Therefore, it is important to have a careful surveillance for potential thyroid neoplasm in patients with c.1159G>A mutation.

The c.2268dup mutation has been reported to be a prevalent mutation in Taiwanese patients with TIOD (Niu *et al.*, 2002; Wu *et al.*, 2002). Most of the CH cases with TIOD were reported to be associated with TPO defects (Ris-Stalpers & Bikker, 2010). These patients originated from Southern China which suggest that the high prevalence of this mutation is due to a founder effect (Niu *et al.*, 2002). The c.2268dup is also a common mutation amongst the dyshormonogenetic CH Malaysian-Chinese patients. However, the c.2268dup was detected in only 27 % of the total alleles studied, lower than what were reported in the Niu *et al*. (2002) (86 %) and Wu *et al*. (2002) (40 %) studies. The patients in this study did not undergo perchlorate discharge test and therefore, they may have CH secondary to defects in other genes. In the Taiwanese

patients, perchlorate discharge test was done and patients with TIOD (most likely have *TPO* gene defects), were screened for *TPO* gene mutations. This could be the reason for the higher prevalence of the c.2268dup mutation in the Taiwanese patients. Nonetheless, the difference in the origin between the Chinese population of Malaysia (Lee & Tan, 1999) and Taiwan (Hsiau, 2000) may also contribute to this variation.

The c.2268dup mutation leads to a PTC at codon 757 (p.Glu757X). Previous study has suggested that mutations that generate a PTC can disrupt the reading frame and thus result in the production of alternatively spliced mRNAs through a nonsense-associated altered splicing (NAS) mechanism (Frischmeyer & Dietz, 1999). Alternatively, others showed that abnormal splicing mechanism can also be triggered as a result of disruption of ESE-binding site sequence by a mutation (Cartegni *et al.*, 2002; Valentine, 1998). In this study, the alternatively spliced TPO transcript with an insertion of 34 bp between exon 12 and exon 13 is postulated to be generated as a result of the interruption of the ESE-binding site sequence in exon 13 of the *TPO* gene. The alternative splicing mechanism triggered by the c.2268dup has unmasked one of the potential acceptor splice sites that is located 46 nucleotides upstream of the 5' splice site of intron 12. Sometimes, the alternative splicing mechanism might help to restore the normal reading frame disrupted by a nonsense mutation (Abramowicz *et al.*, 1992; Ricketts *et al.*, 1987). However, the alternatively spliced mutant TPO transcript that was generated in this study did not restore the normal reading frame but indeed has led to another stop codon after the 739th amino acid. Despite the activation of a potential splice site in exon 13, the natural 3' splice site remains favourable for splicing activity.

In accordance to the mRNA surveillance mechanism, the majority of nonsense-associated transcripts will be degraded via endogenous nonsense-mediated mRNA decay (NMD). However, studies showed that only PTCs that are located 50 - 55 nucleotides upstream of the 3'-most exon-exon junction mediate mRNA decay. The activation of NMD is believed to limit the expression of abnormal transcript and thus protect the cell from possible harmful effect of the encoded truncated protein (Brogna & Wen, 2009; Chang *et al.*, 2007; Maquat, 2005; Sun & Maquat, 2000; Weischenfeldt *et al.*, 2005). In this study, the two PTCs caused by the c.2268dup mutation fulfill the requirement for the mRNA decay, where they are located respectively, at 116 and 201 nucleotides away from the 3' splice site of exon 13 (c.2368).

Theoretically, the expression of normally and alternatively spliced mutant transcripts which was reflected through the total amount of PCR amplicon of exons 12/13 should be similar to the expression of the total TPO transcripts that was represented by the amplicon of exons 4/5. However, in this study, it was found that the TPO transcripts that contained PTCs were found to be less abundant compared to the total TPO transcripts. Further analysis showed no evidence of the remaining unidentified TPO transcripts belonged to TPO6 which does not contain exons 10, 12, 13, 14 and 16. To the best of our knowledge, there is no other similar TPO transcript that has been reported so far. Therefore, the abundant amount of unidentified transcripts is believed to be due to 1) partially degraded PTC-containing TPO transcripts or 2) the increase in shorter alternatively spliced TPO isoforms. Under both circumstances, the reduction of functional TPO transcripts could subsequently lead to insufficient TPO protein translation.

In general, the amount of the unidentified TPO transcripts in the lesion area of thyroid tissue of both patients was higher than that of the normal area. The expression of TPO protein in the lesion area of thyroid tissue of both patients was significantly lower than that of the normal area suggesting that the abundant unidentified TPO transcripts were probably degraded and did not get translated, consistent with the hypothesised mRNA surveillance mechanism. The down-regulation of TPO expression in lesion area of thyroid tissue and disruption of TPO mRNA maturation have been suggested to be associated with malignant neoplasm (Di Cristofaro *et al.*, 2006). Interestingly, when comparison is made between CHP33 and her sister, III-1, the expression of all known TPO transcripts and the corresponding protein was significantly higher in the former than the latter suggesting that the severity of NMD increases with age. In addition, majority of expression profiling studies have reported that lower expression of *Tg* (Karger *et al.*, 2006; Ringel *et al.*, 2001), *TSH-R* (Brabant *et al.*, 1991; Bronnegard *et al.*, 1994; Macchia, 2000; Sheils & Sweeney, 1999) and *NIS* (Passon *et al.*, 2012; Sodre *et al.*, 2008) is often associated with thyroid carcinoma or poorly-differentiated carcinoma cases. In this study, apart from the *TPO*, differential expression of *Tg*, *TSH-R*, and *NIS* between normal and lesion areas of thyroid tissues in CHP33 and her sister was investigated. In summary, both CHP33 and her sister have their own patterns of gene expression. Only the *NIS* in thyroid lesion of CHP33 and *TSH-R* in thyroid lesion of her sister showed a lower expression than that of the normal area. Meanwhile, the *Tg* and *TSH-R* in thyroid lesion of CHP33 and *Tg* and *NIS* in thyroid lesion of her sister (III-1) either showed a higher expression than that of the normal area or was not significantly regulated. The lower expression of *TPO* and *NIS* or *TSH-R* in these two patients may increase the risk of their benign lesion, if the thyroid was not removed, to develop into carcinoma.

In the early part of this study, a smaller size TPO protein in native form was successfully detected in both patients using MoAb47 antibodies which recognises an epitope that is located in between amino acid sequence 713 - 721 (Finke *et al.*, 1991). However, the source of the TPO protein remained unclear since similar size protein can be translated from both normally spliced and alternatively spliced TPO transcripts. The mutant TPO detected in thyroid cells of CHP33 and her sister did not show any enzymatic activity despite the retainment of it's important functional sites, suggesting that the absent region is not only important to serve as a hinge for insertion to the membrane but also plays an important role in proper protein folding. The finding of immunodetection analysis in this study revealed that the monoclonal antithyroperoxidase (TPO) antibody (MoAb47) can also be used to detect the rat TPO protein (Uniprot reference number: P14650). Alignment of the TPO amino acids between humans and rats indicated the full conservation of all residues that are located within the epitope sequence (residues 713 to 721) except for Glu-716 suggesting the Glu-716 is not required in epitope recognition of MoAb47 (Appendix H).

Around 33 % of Malaysian-Chinese patients (or 27 % of the total alleles studied) with dyshormonogenetic CH cases in this study were associated with c.2268dup mutation. The possible degradation of the c.2268dup mutant TPO transcript through NMD and the loss of protein enzymatic activity should be considered as the main factors that caused dyshormonogenetic CH in these patients. In addition, studies have shown that cases of thyroid carcinoma can arise from congenital goitre that is associated with *TPO* mutation (Chertok Shacham *et al.*, 2012; Medeiros-Neto *et al.*, 1998). Findings from these studies suggest that the *TPO* mutations do not only cause thyroid dyshormonogenesis but could possibly be involved in malignant transformation of the goitrous thyroid gland. Considering all the subjects in this study with

homozygous c.2268dup except CHP59 who was 12-year old, have developed goitre/MNG at mid or late adolescence, it is important to have a careful surveillance for potential thyroid neoplasm in these patients.

Apart from the c.2268dup mutation, a novel c.670_672del mutation in exon 7 of the *TPO* gene was detected in patient CHP58. Further analysis showed that the mutation was not detected in 100 chromosomes from 50 normal unrelated individuals and thus it is believed that the novel alteration is a disease-causing mutation. The parents of the patient who were either heterozygote for c.2268dup or c.670_672del mutations were reported to be asymptomatic suggesting that the CH in CHP58 is caused by a compound heterozygous mutation in the *TPO* gene.

The deletion of 3 nucleotides (GAC) is predicted to produce an in-frame deletion of a single amino acid, aspartic acid (p.Asp224del), in the TPO protein. Three dimensional model analysis of the mutant protein revealed that the deleted Asp-224 residue is located within a beta-strand. The mutation has brought conformational changes to the protein by shortening the length of the beta-strand and also disrupting the formation of trifurcated hydrogen bonds between Asp-223 with Asp-224, Arg-225 and Tyr-226. It is also predicted that the deletion of Asp-224 will influence the formation of a salt bridge between Asp-223 and Arg-225. Since the altered sites are located so close to His-494, a proposed iron (heme axial ligand) binding site (Bikker *et al.*, 1997), it could possibly interfere with binding of the iron ion at His-494 or the electron transfer activity of TPO where His-494 is the source of the electron (Nordlund, 2011).

Interestingly, three aspartic acid residues: Asp-222, Asp-223 and Asp224 present in the same beta-strand that is located on the outer surface of the wild type TPO contribute to a highly negatively-charged region, suggesting the deletion of Asp-224 decreases the solubility of the protein in water. In addition, the negatively-charged region is also conserved across many species including mice and rats implying that this region is crucial for the normal activity of the protein.

In summary, the deletion of Asp-224 in TPO protein is postulated to 1) cause structural instability and/or 2) interfere with the binding of the metal ion at His-494 and/or 3) influence the electron transfer activity. Apart from the possible effects of the mutation on protein activity, the possibility of the mutation causing abnormal splicing cannot be excluded since c.670_672del was shown to interrupt the binding site of a few ESE proteins. The combination of Asp-224 mutation with a nonsense mutation in exon 13 suggests that both alleles give rise to an almost inactive TPO enzyme.

Most cases of CH associated with defects in the *TPO* gene were caused by either homozygous or compound heterozygous mutations. In the present study, three different mutations were identified in CHP38 with dyshormonogenetic CH. One of the mutations, c.1186C>T which is located in exon 8, has yet to be reported and it was not detected in 100 chromosomes from 50 normal unrelated individuals suggesting that the alteration is a disease-causing mutation instead of a polymorphism.

Findings from the current study showed that the c.1186C>T mutation occurs within a sequence recognised by splicing regulators and therefore can possibly interfere with the splicing activity of TPO pre-mRNA. As discussed before, exon 8 of the *TPO* gene is significantly important for the synthesis of a functionally active protein. Hence,

the disturbance in the splicing regulators-binding site can give rise to the production of various TPO protein isoforms which might not have enzymatic function.

The c.1186C>T is expected to cause a substitution of arginine to cysteine at codon 396. Multiple sequence alignment showed complete conservation of Arg-396 amongst all animals examined in this study implying this residue is important in the structure/function of the TPO. A study has shown that the Arg-396 is one of the important amino acids which could be involved in stabilising the transition state of TPO protein during the catalytic intermediate formation (Taurog & Wall, 1998). The formation of a stable catalytic intermediate (compound I) of the *TPO* with H_2O_2 is crucial for thyroid hormone synthesis (Magnusson *et al.*, 1984a, 1984b). Previous studies suggested that the catalytic process is initiated by the diffusion of H_2O_2 into the active site of the TPO protein. The α-nucleophile H_2O_2 donates a proton to the distal imidazole ring (His-239) to form a bond with the iron ion bound to residue His-494. After binding takes place, the protein attains transition state to form compound I. The arginine at position 396 is believed to play a role in stabilising the charge for transition state of the protein through electrostatic interaction (Dunford, 2001; Phillips *et al.*, 1990; Taurog & Wall, 1998). Alternatively, it is believed that the arginine contributes to the abnormally low pKa value of the distal histidine in the native resting enzyme. The changes of the pKa value in the transition state of the distal imidaloze are the key to determine the effectiveness of the catalysis process/rate of the compound I formation (Dunford, 2001). Therefore, a substitution from arginine to cysteine can bring devastating effects to the protein stability.

In the present study, the 3-D model analysis showed that the p.Arg396Cys mutation has led to the structure alteration through the modification of the hydrogen bond network in the hydrophobic pocket which might interfere with the heme binding. Furthermore, the replaced residue, cysteine, at codon 396 might possibly form a new disulphide bond through its thiol group which can affect the folding and stability of the protein.

Apart from the c.2268dup and c.1186C>T mutations, a c.2647C>T mutation was identified in exon 16 where it is predicted to lead to a substitution of proline to serine at codon 883 in the C-terminal tail (Val-869 to Leu-933) of the TPO protein. Interestingly, this mutation had also been reported in populations of Korea (Lee *et al.*, 2011) and Japan (Umeki *et al.*, 2004). Whether the c.2647C>T mutation is possibly a founder mutation is not known. The founder effect of this mutation is required to be confirmed by a larger sample size of unrelated patients in East Asia populations.

To date, very little is known about the function of the intracellular C-terminal domain of the TPO. Comparison of amino acid sequence of human TPO with other species revealed that proline at codon 883 is not well conserved suggesting that the Pro-883 is probably not critical for structure/function of the TPO. This postulation is further strengthen by the findings from SIFT and Polyphen-2 analyses which indicates that this mutation is harmless and does not modify TPO function. The 3-D model analysis of the c.2647C>T mutation in the present study could not confirm the exact structural modifications on the domain due to lack of suitable template for models building.

Despite the *in silico* findings above indicating that the substitution of Pro-883 to serine might not be critical to the protein structure/function, previously reported cases showed that patients associated with this mutation presented with severe dyshormonogenetic CH (Lee *et al.*, 2011; Umeki *et al.*, 2004). Since HSF analysis predicted that the c.2647C>T mutation interrupts the sequences recognised by ESE proteins, therefore, we postulate that the sequence alteration could possibly regulate the splicing activity of the TPO pre-mRNA and lead to CH in these patients.

Due to the inavailability of blood sample from CHP38's parents who were reported to be physically healthy and CH asymptomatic, therefore, we could only speculate that both parents carried mutation(s) in a single allele. It is worth to note that many studies have shown that multiple mutations in a patient could lead to "dosage effect," contributing to a more severe illness (Bernard *et al.*, 2008; Kelly & Semsarian, 2009; Piippo *et al.*, 2001; Sethuraman *et al.*, 2007). The combination of the three mutations c.1186C>T, c.2268dup and c.2647C>T, suggesting both alleles give rise to a functionally inactive TPO enzyme. Therefore, careful long-term follow-up of patients with multiple mutations is needed to ensure that the hypothyroid state does not develop into more serious thyroid disorders and the patients can benefit from earlier additional specific treatment.

In the present study, another novel, homozygous mutation, c.1502T>G, was identified in exon 9 of the *TPO* gene in two siblings (CHP53 and her sister, II-2) who had severe goitrous CH. Family members with heterozygous p.Val501Gly were asymptomatic supporting the recessive inheritance pattern (Park & Chatterjee, 2005). The mutation was not detected in 100 chromosomes from 50 normal unrelated

individuals suggesting that the c.1502T>G alteration is disease-causing instead of a polymorphism.

The sequence alteration caused by the c.1502T>G mutation is predicted to interrupt the ESE-binding site sequence which could possibly give rise to production of a various TPO isoforms due to an activation of alternative splicing mechanism. Exon 9 of the *TPO* gene has been suggested to code for a site that participates in catalytic mechanism and therefore any modifications of this critical region can lead to production of enzymatic inactive TPO protein.

Val-501 is conserved amongst many animals including mice and rats implying its importance in the structure/function of the TPO. Valine is seldom directly involved in protein function due to its very non-reactive side chain (Betts & Russell, 2003). Therefore, alteration of valine residue at codon 501 is more likely to affect the protein conformation. Moreover, replacing valine with glycine might lead to less hydrophobic interactions as the former has a larger aliphatic side chain while the latter has hydrogen as its side chain. Nonetheless, 3-D modeling showed structural alterations in the TPO mutant, suggesting the mutation could cause structural instability of the protein.

A proposed iron (heme axial ligand) binding site, His-494 (Bikker *et al.*, 1997), is located six residues away from the mutation site. The early step in the biosynthesis of thyroid hormones involves the oxidation of I^- to iodinium (I^+) ions by TPO in the presence of H_2O_2 (Lagorce *et al.*, 1991). The heme iron in the TPO plays an important role in the redox reaction. In this study, 3-D model analysis showed that the iron binding site of the p.Val501Gly mutant TPO is slightly exposed than that of the wild type. It is very likely that the additional entry in the proposed-binding pocket of mutant

TPO would interfere with the binding of the ligand and/or causes improper conformational changes after binding. Alternatively, the absence of a relatively bulky aliphatic side chain, due to the replacement of valine by glycine, could alter the hydrophobic pocket and thus interfere with the heme binding at Glu-399. Interestingly, Rivolta *et al.* in 2003 reported that p.Pro499Leu mutation in the TPO which is also located within the hydrophobic pocket was associated with severe goitrous CH. Therefore, this region is believed to be significantly important for the normal activity of the protein.

The mechanism by which p.Val501Gly in the TPO may induce MNG remains to be elucidated, although the structure of the protein appeared to be modified. However, it should be noted that the structural modifications in the mutant might lead to new protein functions which could subsequently contribute to the goitre development with an increase in thyroglobulin level, indicating increase in thyroid mass.

Previous studies reported that the presence of SNPs can alter the risk of developing a disease (Han *et al.*, 2012; Li *et al.*, 2010; Singh *et al.*, 2011). It is believed that the presence of SNPs can be associated with the changes in the expression levels of target genes or promote alternative splicing in gene transcripts (Cheung *et al.*, 2005; Lalonde *et al.*, 2011). In this study, general transcription factors binding sites including the putative TATA box, CAAT and GC box were detected within 50 bp upstream from the transcription start site of the *TPO* gene. The essential role of the GC box in mediating transcription has been demonstrated in many studies (Charron *et al.*, 2003; Hapgood *et al.*, 2001; Lv *et al.*, 2009). Therefore, the alteration in the GC box due to the novel c.1-192C>A polymorphism could possibly affect the transcription initiation process and thus alter the expression levels of *TPO* gene in an individual. Apart from

the GC box alteration, the c.180-6C>A polymorphism found in within the 5' splice junction of the exon 4 was shown to reduce the intrinsic strength of the natural splice site which could subsequently lead to aberrant splicing through the triggering of other potential acceptor splice sites. It is not known whether the c.1-192C>A or c.180-6C>A polymorphisms can lead to increased risk of dyshormonogenetic CH in an individual. However, findings from other studies suggested that the cumulative effects from the SNP-SNP interaction can confer susceptibility to a disease (Borroni *et al.*, 2006; Pezzini *et al.*, 2005; Vogelsang *et al.*, 2012).

Although the present study has reported some preliminary findings, there are several limitations need to be noted. The main limitation concerns the factor of thyroid tissue specimen collection in our patients. Most of the patients in this study have yet to undergo surgery when this study was carried out. Therefore, it is difficult to compare and confirm the enzymatic activity of TPO in these patients since the *TPO* gene is only expressed specifically in the thyroid gland (Chedrese, 2009). In addition, studies thus far have not reported the full length of TPO1 mRNA transcript (3152 bp) that can be detected in either blood or thyroid tissue through RT-PCR method. Consistent with this, the attempt to detect full length TPO1 in blood and thyroid tissue using RT-PCR method in this study was not successful (data not shown). This problem could be due to the various transcripts resulting from alternative splicing that possess different lengths of the poly(A) tail (Ferrand *et al.*, 2003; Zhao *et al.*, 1999). Random hexamers and oligonucleotides primers used during reverse transcription process might not be able to detect some of the transcripts and hence cause the TPO mRNAs extracted from thyroid tissue not reverse-transcribed into cDNA efficiently.

In line with the recessive mode of inheritance, the findings in this study revealed that only individuals associated with either homozygous or compound heterozygous form of *TPO* mutation were affected with dyshormonogenetic CH whereas those with one mutant allele remained asymptomatic. Mutation in the *TPO* gene is one of the underlying genetic factors that causes hereditary dyshormonogenetic CH in this Malaysian cohort of patients.