A Homozygous *TPO* Gene Duplication (c.1184_1187dup4) Causes Congenital Hypothyroidism in Three Siblings Born to a Consanguineous Family

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Abstract

Congenital hypothyroidism (CH) is the most common neonatal endocrine disease, and germ-line mutations in the *TPO* gene cause the inherited form of the disease. Our aim in this study was to determine the genetic basis of congenital hypothyroidism in three affected children coming from a consanguineous Turkish family. Because CH is usually inherited in autosomal recessive manner in consanguineous/multicase families, we adopted a two-stage strategy of genetic linkage studies and targeted sequencing of the candidate genes. First, we investigated the potential genetic linkage of the family to any known CH locus, using microsatellite markers, and then screened for mutations in linked-gene by conventional sequencing. The family showed potential linkage to the *TPO* gene and we detected a homozygous duplication (c.1184_1187dup4) in all cases. The mutation segregated with disease status in the family. This study confirms the pathogenicity of the c.1184_1187dup4 mutation in the *TPO* gene and helps establish a genotype/phenotype correlation associated with this mutation. It also highlights the importance of molecular genetic studies in the definitive diagnosis and accurate classification of CH.

Keywords
- TPO
- mutation
- genetics
- duplication
- congenital hypothyroidism
- thyroid dyshormonogenesis

Introduction

Congenital hypothyroidism (CH) is the most common neonatal endocrine disorder with an incidence of 1/3,500 live births, and causes mental retardation and growth delay unless a timely and proper treatment is introduced.[1] Approximately 85% of CH cases are sporadic and approximately 2% is familial having a known genetic cause. To date, 11 causative genes have been described for the pathogenesis of inherited CH.[2] ►**Table 1** shows the details of all these loci and associated clinical phenotypes. Some of these genes (e.g., *TSHR* and *PAX8*) are associated with primary thyroid dysgenesis (CHNG)[3,4] whereas some (e.g., *TPO* and *TG*) are with thyroid dyshormonogenesis (TDH).[5] Currently there are seven genes known to cause congenital TDH, which encode for proteins involved in thyroid hormone biosynthesis.[6] Major steps in thyroid hormone synthesis include iodide transfer from blood to thyrocytes and then into the follicular lumen, oxidation, and covalent linkage to tyrosine residues of thyroglobulin (TG) and, upon their hydrolysis, eventual coupling of iodinated tyrosyl residues into iodothyronines (T_4 and T_3).[7]

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Table 1 Genes causing congenital hypothyroidism, associated phenotypes, and microsatellite markers used for their linkage analysis

Gene	Ch locus	Phenotype[a]	Microsatellite markers
NIS	19p13.2	TDH1	D19S566, D19S593 D19S103, D19S898
TPO	2p25	TDH2A	D2S2980, D2S323 D2S1780, D2S2245
PDS	7q31	TDH2B	D7S2459, D7S692 D7S2456, D7S799
TG	8q24	TDH3	D8S1740, D8S256 D8S1746, D8S558
DEHAL1	6q25.1	TDH4	D6S1654, D6S440 D6S1687, D6S960
THOXA2	15q21.1	TDH5	D15S100, D15S123 D15S132, D15S977
THOX2	15q21.1	TDH6	D15S100, D15S123 D15S132, D15S978
TSHR	14q31	CHNG1	D14S1433, D14S606 D14S1008, D14S610
PAX8	2q12-q14	CHNG2	D2S2269, D2S160, D2S410, D2S1893
TSHB	1p13	CHNG4	D1S2756, D1S2881, D1S2852, D1S189
NKX2–5	5q34	CHNG5	D5S400, D5S2075, D5S211, ATA52D02

Abbreviation: Ch, chromosomal.
[a]As described in OMIM database: TDH, thyroid dyshormonogenesis; CHNG, congenital nongoitrous hypothyroidism.

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Thyroid peroxidase (TPO) enzyme catalyzes the oxidation and organification of iodide, which requires hydrogen peroxide (H_2O_2) as the final electron acceptor.[8,9] Therefore, the generation of H_2O_2 is a critical step in the synthesis of thyroid hormones.[10] Previous reports have found that a defect in the system that generates H_2O_2 causes CH.[11–13] Two dual oxidases (DUOX1 and 2) have recently been identified as the components of thyroid H_2O_2 generating system.[14,15] TPO is a thyroid-specific heme peroxidase localized in the apical membrane of thyrocytes and plays a central role in thyroid hormone biosynthesis by catalyzing (1) oxidation of iodide; (2) its organification (binding to tyrosine residues of thyroglobulin); and also (3) coupling reactions of inert monoiodotyrosine and diiodotyrosines to form the active thyroid hormones T_4 and T_3.[2] As far the other genes involved in thyroid hormone synthesis, mutations in *TPO* causing permanent CH are mostly inherited in an autosomal recessive fashion and to date more than 60 distinct mutations have been described in this gene.

To investigate genetic background of CH, we developed a two-tier strategy combining genetic linkage studies and full sequencing of candidate genes in familial cases and identified several mutations to date in different CH genes.[16–26] In the current study we aimed to determine genetic cause of CH in a consanguineous family with three affected siblings. Here we report a homozygous duplication (c.1184_1187dup4) in the *TPO* gene detected in all cases and associated clinical phenotypes. Molecular genetic analyses facilitate definitive diagnosis and accurate classification of CH in familial cases.

Materials and Methods

Subjects

Three cases born to a consanguineous Turkish family were ascertained through our studies on the genetics of CH.[16–26] The older sister was first diagnosed at the age of 8 months with hormone values of thyroid-stimulating hormone (TSH) 132 mIU/L (normal 0.35–5.5), free thyroxine (fT_4) 0.3 ng/dL (normal 0.8–2.0), and T_3 20 ng/dL (normal 80–120). Thyroid ultrasonography showed a thyroid gland of normal size and location. Her growth parameters were between 3rd and 10th percentile. Perchlorate discharge test at age 9 indicated partial iodide organification defect (PIOD). Test values were 1st hour: 1,600 cpm, 2nd hour: 2,200 cpm (before iodide), and 30': 700, 60': 650, 90': 560, 120': 500, 180': 450. Currently at the age of 26, she has a moderate mental retardation and is euthyroid with 150 µg/day L-thyroxine treatment. Her younger brother with the same mutation was born at term and first diagnosed at 45 days of age through investigations for prolonged jaundice. His hormone values at diagnosis were TSH 128 mIU/L, fT_4 0.4 ng/dL (normal 0.8–2.0), and T_3 15 ng/dL (normal 80–120). Thyroid ultrasonography performed at 2 months of age showed diffuse hyperplasia and perchlorate discharge test indicated PIOD. Test values were 1.saat:1,400 cpm, 2.saat:1,500 cpm (before iodide), and 30': 450 cpm, 60': 40 cpm, 90': 350 cpm, 120': 300 cpm, 180': 300 cpm. Upon detecting multiple solid nodules in both lobes, he was operated at the age of 18 and pathology report indicated dyshormonogenetic goitre including multiple cellular proliferative nodules. Currently, at the age of 23, he is euthyroid with 100 µg/day L-thyroxine treatment and has a borderline IQ level. Their youngest brother was diagnosed at 9 days of age with a neonatal TSH level of 140 mIU/L. A repeat test showed a plasma TSH level of 470 mIU/L and an fT_4 level of less than 0.4 ng/dL. Ultrasonography indicated thyroid hyperplasia. Currently, at the age of 8, he is euthyroid with 62.5 µg/day L-thyroxine treatment and his developmental milestones have been normal. The treatment was introduced at the time of diagnosis in all cases, which differed between them. Compliance with the treatment was also variable among cases. Their parents are healthy and free of any signs or symptoms of hypothyroidism. Informed consent was

obtained from the family and venous blood samples were collected from all family members. All procedures performed were in accordance with the Declaration of Helsinki and the study was approved by relevant IRBs/Ethics Committees. DNA was extracted by using standard methods and stored at −20° C until analyzed.

Methods

Linkage Analysis

First we performed linkage analysis to all 11 known CH loci in all family members using microsatellite markers. Four primer pairs surrounding each locus were selected (►**Table 1**). Fluorescent labeling of one oligonucleotide of each primer pair enabled the sizing of polymerase chain reaction (PCR) products in a capillary electrophoresis machine by the use of GeneMapper v4.0 software suite (Applied Biosystems, Warrington, UK). By combining genotypes for each microsatellite marker, we constructed haplotype tables for each family member. As autosomal recessive inheritance was assumed in consanguineous families, homozygosity of a particular haplotype for a locus in cases accompanied by heterozygosity of the same haplotype in both parents was taken as suggestive of linkage to that locus.

Direct Sequence Analysis of the *TPO* Gene

The DNA template of the *TPO* gene was downloaded from the Ensembl database (ENSG00000115705). All alternative transcripts (17 in total) were included to ensure that primers were designed to cover all coding exons and intron/exon boundaries. Intronic primers flanking the coding sequence were designed for PCR amplification using ExonPrimer and Primer3. Primer sequences and PCR conditions are available upon request. PCR products were size-checked on 1% horizontal agarose gels and cleaned up using MicroCLEAN (Microzone, Haywards Heath, UK) or gel-extracted using QIAquickTM Gel Extraction kit (Qiagen, Crawley, UK). The purified PCR products were sequenced in both forward and reverse directions using the ABI BigDye Terminator v3.1 Cycle Sequencing kits on an ABI Prism 3730 DNA Analyzer (Applied Biosystems, Warrington, UK). Analyzed sequences were then downloaded using Chromas software and assessed for the presence of alterations.

Mutation Screening for All Other Known Causative *CH* Genes by Next-Generation Sequencing

To exclude any other causative mutations in other *CH* genes, we chose Illumina's TruSeq Custom Amplicon (TSCA) assay for next-generation sequencing (NGS) library preparation (Burrington, UK). Illumina Design Studio (http://designstudio.illumina.com/) was used to create the optimal TSCA panel design for 16 selected *CH* genes, 11 known causative (►**Table 1**) and 5 of our own candidates. Prior to target enrichment, the selected samples were diluted to 25 ng/µL, with calculations based on average DNA concentration from duplicate Qubit assays. Samples of concentration less than 25 ng/µL (but ≥ 16.7 ng/µL) were used neat, with volume increased from the standard 10 µL up to a maximum of 15 µL (such that the recommended 250 ng DNA was added, as for all samples). Target enrichment was performed using the designed TSCA kit, in accordance with manufacturer's instructions (Illumina TruSeq Custom Amplicon Library Preparation Guide. Part # 15027983). TSCA libraries were sequenced on the in-house Illumina MiSeq platform, using a 500-cycle reagent kit and paired-end sequencing (2 × 251 cycle reads). In accordance with manufacturer's instructions (Illumina MiSeq System User Guide. Part # 15027617), the diluted Amplicon Library was transferred into the reagent cartridge, and this was loaded onto the MiSeq alongside the Illumina-provided flowcell and buffer. Initial data analysis was automatically performed on-instrument (MiSeq Reporter software, TruSeq Amplicon Workflow); which demultiplexes indexed reads, generates FASTQ files, and performs alignment. Analysis of coverage and variant calling was performed using SoftGenetics NextGENe v2.3.3. For the positive controls, NextGENe's Mutation Report and sequence alignment view were used to examine calling and coverage of all known variants. Finally, NextGENe's Variant Comparison Tool was used for each run to generate a list of all called variants across samples.

Results

Linkage to the *TPO* Gene

First linkage analysis using microsatellite markers was performed in all family members and haplotype tables were constructed for each family member by combining the scores for each marker to observe the segregation of the genotype along with the disease status. The linkage analysis using these tables indicated a potential linkage to the *TPO* locus in the family; that is, all CH cases were homozygous for a disease associated haplotype while both parents were heterozygous for the same haplotype (►**Fig. 1**). These results suggested that the disease-associated haplotype segregated with the disease status in the family assuming autosomal recessive inheritance model that is the most likely pattern in consanguineous families.

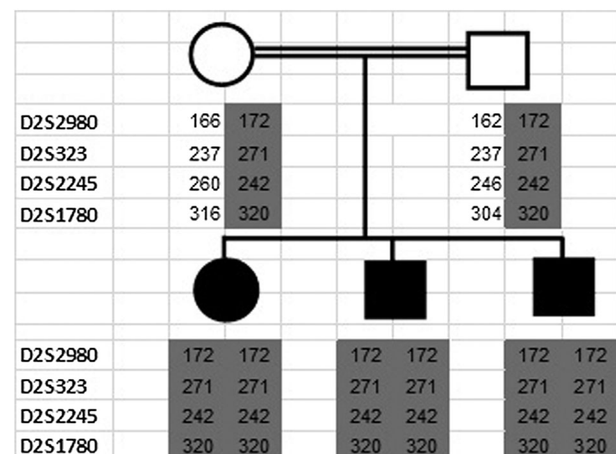

Fig. 1 The scores of microsatellite marker analysis surrounding the *TPO* locus in family members.

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Mutation Detection in the *TPO* Gene

Upon finding potential linkage to the *TPO* locus, we proceeded to sequence the entire coding region (and flanking sequences) of the *TPO* gene in all members of the family. Direct sequencing analysis revealed a homozygous duplication of 5′–GGCC–3′ tetranucleotide in exon 8 of the *TPO* gene (c.1184_1187dup4) in all cases, which creates a frameshift and results in a premature stop codon in exon 9. Both parents carried the mutation at heterozygous state, which was consistent with the linkage results. The mutation was not present in 400 ethnically matched control chromosomes. Neither case nor the other family members carried any other mutation in the *TPO* gene.

The Exclusion of Mutations in Other Causative *CH* Genes

With the availability of NGS, we developed a comprehensive NGS-based strategy for genetic diagnosis of CH as described elsewhere.[21] This test included full sequencing of all 11 known causative *CH* genes and 4 of our own strong candidate genes. Mutation analysis of these genes revealed no mutations in any of these genes, neither in the case nor in any other family members. These results implicated that c.1184_1187dup4 in the *TPO* gene was only mutation to cause the disease in the family.

Discussion

Congenital hypothyroidism (CH), if untreated, might cause severe developmental delay and genetic defects have long been indicated in the etiology of the disease. Currently improving genetic analyses provide a powerful tool to unravel the pathogenesis of the disease, and as the number of causative genes grows, underlying molecular mechanisms become clearer in increasing number of patients. This is especially important for TDH phenotype as it is often inherited autosomal recessively where both parents usually are healthy carriers of a mutation in a particular causative gene.

The *TPO* gene is located on chromosome 2p25 and covers approximately 150 Kb of DNA.[27,28] It is composed of 17 coding exons with a 3048 nucleotide-full-length transcript, which encodes 933-amino acid TPO enzyme. The *TPO* gene alteration detected in this study (c.1184_1187dup4) is located in exon 8 of the gene and causes a frameshift, which results in stop codons in exon 9. On the other hand, this mutation also causes the activation of a cryptic splice site within the exon 8. The c.1184_1187dup4 mutation was first described by Abramowicz et al.[29] Normally the functional consequence of the mutation is expected to be deleterious, but the alternative splicing might represent a salvage mechanism because it restores the normal reading frame disrupted by the mutation. Although no enzymatic activity can be expected from the normal splicing product of the mutated gene, the product of alternative splicing of the mutated gene might have some residual TPO enzyme activity.

In terms of phenotypic presentation, all cases in our study showed PIOD, which confirms the residual TPO activity in their thyroid glands. Therefore, it is plausible to suggest that c.1184_1187dup4 mutation leads to a partial loss of function (LOF), and that there is a good degree of genotype/phenotype correlation associated with this mutation. However, clinical presentation in other cases with this mutation could be modified with iodine status while the phenotype in cases with total LOF mutations is invariably represented with total iodine organification defect (TIOD). Therefore the phenotype in these cases is more likely to be variable. The reason for the youngest case having normal developmental milestones while older cases having mental retardation might be earlier introduction and better compliance with treatment in the last case compared with the previous cases. These differences in treatment might also explain other phenotypic variability between the patients. In contrast, total LOF mutations invariably lead to TIOD and thus allow the establishment of more consistent genotype/phenotype correlations.

While only 15% of sporadic CH is caused by defects in thyroid hormone synthesis, most of the familial cases result from TDH inherited autosomal recessively. We previously reported *TPO* mutations as the most common cause of familial TDH,[22] and thus *TPO* mutation analysis takes a major place in determining the etiology the disease in such cases. Because mutations in other *TDH* genes such as *DUOX2*, *DUOXA2*, *TG*, and *IYG* are more likely to be associated with PIOD, especially in cases with TIOD screening for *TPO* mutations should be the first line of investigation. A prior linkage analysis in familial cases could further ensure if *TPO* mutation analysis would be feasible.

Here we conclude that CH in our cases was caused by c.1184_1187dup4 *TPO* mutation, and that this mutation is associated with PIOD phenotype. Our study contributes to the establishment of a firm genotype/phenotype relationship associated with this mutation. Molecular genetic studies as such would allow the description of exact etiology and pathogenic mechanism of the disease in familial CH cases.

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