MUTATION IN BRIEF

Five Novel Inactivating Mutations in the Thyroid Peroxidase Gene Responsible for Congenital Goiter and Iodide Organification Defect

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Thyroid peroxidase (TPO) defects, typically transmitted as autosomal recessive traits, result in hypothyroid goiters with failure to convert iodide into organic iodine. We analyzed the TPO gene in 14 unrelated patients with clinical evidence of iodide organification defects. Seven of the affected individuals harbored mutations in the TPO gene; one was compound heterozygous, the others were simply heterozygous for TPO mutations. Five novel mutations have been identified, one of which was found to be a single nucleotide deletion, while the other four were single nucleotide substitutions. A frameshift mutation c.387delC was detected in exon 5 which leads to an early termination signal in exon 7 (p.N129fsX208). Two missense mutations were identified in exon 8. The first, a c.920A>C transversion that results in a p.N307T substitution, was found in two patients. The second, a c.1297G>A transition, results in p.V433M. A c.1496C>T transition was detected in exon 9 that caused the substitution p.P499L. Finally, in exon 14 a c.2422T>C transition was identified, causing a p.C808R change. In addition, the previously reported GGCC duplication in exon 8 (c.1186_1187insGGCC; p.R396fsX472) was also detected in two affected individuals, one of whom was a compound heterozygous (p.R396fsX472/p.V433M).

KEY WORDS: hypothyroidism; goiter, congenital; TPO; mutation screening

INTRODUCTION

Thyroid peroxidase (TPO; MIM# 606765) is the enzyme responsible for the iodination and coupling of specific tyrosine residues in thyroglobulin (Medeiros-Neto et al., 1993). The single gene for TPO (GenBank Accesion Number: NT_033000) is located on chromosome 2 spanning 150 Kb of genomic DNA and contains 17 exons (Kimura et al., 1989). Full length TPO mRNA (GenBank Accesion Number: NM_000547) is 3.1 Kb and encodes a

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protein of 933 amino acids. Defective biosynthesis of thyroid hormone by aberrant TPO expression (MIM# 274500) is one of the causes of thyroid dyshormonogenesis. This type of primary congenital hypothyroidism is typically characterized by total perchlorate discharge test, indicating a defect in converting accumulated iodide to organically bound iodine (Medeiros-Neto et al., 1993). The first reported mutation in the TPO gene was a homozygous GGCC insertion-duplication at position 1186 in the eighth exon of the TPO gene (c.1186_1187insGGCC, p.R396fsX472; Abramowicz et al., 1992). The resulting frameshift generates a stop codon in exon 9, which would result in a grossly truncated protein with no expected activity. An alternative splicing by a cryptic acceptor splice site in exon 9 restores the normal reading frame disrupted by the mutation and eliminates the stop codon. de Vijlder and co-workers described numerous inactivating mutations of the TPO gene: missense and nonsense mutations, splicing errors, deletions and insertions of nucleotides (Bikker et al., 1994, 1995, 1996; Bakker et al., 2000, 2001). Several other mutations have been reported (Kotani et al., 1999; Pannain et al., 1999; Santos et al., 1999; Ambrugger et al., 2001; Kotani et al., 2001; Niu et al., 2002; Umeki et al., 2002; Wu et al., 2002). Partial organification defects are also associated with mutations in the PDS (SCL26A4) gene (Everett et al., 1997), causing Pendred's syndrome (MIM# 274600), which is classically characterized by sensorineural hearing loss, goiter and impaired iodide organification. More recently, inactivating mutations in the thyroid oxidase 2 (THOX2, also known as DUOX2) gene were associated with congenital hypothyroidism and partial or total iodide organification defects (Moreno et al., 2002; MIM# 607200).

In the present study screening by Single Stranded Conformational Polymorphism (SSCP) and direct sequencing analysis revealed five novel mutations in the TPO gene, in patients with congenital goiter and iodide organification defects.

MATERIALS AND METHODS

Selection of Patients

From an original cohort of 40 patients with congenital goitrous hypothyroidism at screening, 14 selected patients with iodide organification defect (perchlorate discharge test, 46 percent or more) were studied. Written informed consent was obtained from all individuals involved in this study. Genomic DNA was isolated from white blood cells by the SDS-proteinase K method.

SSCP analysis

SSCP was used to screen for the presence of mutations in each exon of the TPO gene. The complete coding sequence of the human TPO gene was amplified from the affected patients. Polymerase chain reaction (PCR) were performed in 50 µl, using a standard PCR buffer (Invitrogen, Life Technologies, Carlsbad, CA), containing 125 - 250 ng of genomic DNA, 1.5 or 2.5 mM $MgCl_2$, 200 µM of each dNTP, 4 % dimethylsulfoxide, 1 U Taq polymerase (Invitrogen, Life Technologies) and 50 pmol of each forward and reverse primers. Intronic primers were specially designed for each of the 17-TPO exons. Their oligonucleotide sequences and the position of their 5'coding sequence ends are shown in Table 1. Samples were denatured at 95 °C for 3 minutes followed by 40 cycles of amplification. Each cycle consisted of denaturation at 95 °C for 30 seconds, primer annealing at 55-63 °C for 30 seconds, and primer extension at 72 °C for 1 minute. After the last cycle, the samples were incubated for an additional 10 minutes at 72 °C to ensure that the final extension step was complete. The gel matrix for SSCP contained 8 or 10 % polyacrylamide (29:1) (Invitrogen, Life Technologies), with or whithout 10 % glycerol. Samples were electrophoresed for up to 18 hs at a constant temperature (4 °C). DNA was visualized by silver-staining.

DNA sequencing

Samples showing an aberrant pattern in SSCP analysis and exons 8, 9, 10, 12 and 14 of selected patients were directly sequenced with the Taq polymerase-based chain terminator method (*f*mol, Promega, Madison, WI) using the same TPO forward and reverse primers used in the SSCP analysis (Table 1). The results were analyzed using the PC gene sofware program (Intelligenetics, Geneva, Switzerland).

Validation of TPO mutations by SSCP or restriction enzyme analysis

All mutations were validated by studying 50 normal unrelated individuals by SSCP analysis or by digestion of the amplified PCR products with appropriate restriction enzymes, according to the specifications of the manufacturers.

Table 1: Summary of Primers Used for PCR Amplification and Sequencing

Exon	Forward Primers		Fragment size (bp)	Reverse Primers	
	Position of 5' end	Nucleotide sequence (5'→ 3')		Position of 5' end	Nucleotide sequence (5'→ 3')
1	-97	*ctgtccccacgaagaac*	305	+119	gctgagagacgccaccc
2	-117	gtagaggctgcgtggag	306	+94	gtgactctcaggagcta
3	-100	ggcatcaccgcagcaag	259	+74	cacacgtgtgtggatgt
4	-238	tgccattttcctcatca	523	+115	catcctgcttggtctca
5	-21	atggtttcctatttttcacagAT	294	+140	cggggaggccaaggaca
6	-103	ggccccacttattctcc	296	+63	ttccctccctcagcatc
7	-92	gaaccacaccaggaagt	411	+112	tgggaataggacaaaga
8 A	-25	accttgaactcccctttgcc	306	1100	ACGAAGGGCAGGTAGGCG
B	-82	tcgtcgccggcctcgaactt	369	1106	GGCGGCACGAAGGGCAGGTA
C	979	TCCACCGTGTATGGCAGCTC	450	+90	ggagagagaagccacgatgc
9	-139	cagctgaggcccttattaca	415	+17	ggaccgcactcactcacCTC
10	-112	tgggcaacagaaagaacg	343	+60	gctagctcggcaaatcc
11	-27	aaaccctgcagcctctccc	297	+32	cgtgaaggaagacgctctg
12	-44	ttctccatgcactgtgacc	296	+43	atgcacgtgctgtaacgtgg
13	-70	acagggacgttggtgtgtgg	344	+103	tcagaagcacctttggcg
14	-44	tgcagccgcttcctctcacg	271	+95	gatggtgattgacagttgcc
15	-74	agactcaggcaggacaacc	249	+75	gcttcattgcagccatgtcc
16	-42	tgccggaccctctcccgataa	236	+64	gacacccagatcctgtccaa
17	-139	aagaaggatggctcatctcg	285	2853	CTGCTGATTTCCGATTTGCC

Exon sequences are in capital letters, intron sequences are in lower-case letters; promoter sequences are in italic lower-case letters.

The exonic nucleotide position is designated according to TPO mRNA reference sequences (GenBank Accesion Number: NM_000547). The intronic nucleotide position is numbered from the exon end: negative numbers start from the g of the ag splice acceptor site, positive numbers start from the g of the gt splice donor site.

RESULTS AND DISCUSSION

All 17 exons of the TPO gene, including the flanking intronic sequences and the promoter region were screened by SSCP from 14 unrelated patients with iodide organification defect and normal controls. Analysis of PCR products showed four different patterns of migration that were not detected in the normal subjects. Sequence analysis of these variants revealed three novel mutations and one previously identified insertion-duplication (Table 2). One, is a cytosine deletion at nucleotide 387 in exon 5 (c.387delC), resulting in a frameshift at amino acid 129 with a premature stop at 208 in exon 7 (p.N129fsX208). The second, found in two patients, is a missense mutation in exon 8. It is an adenine to cytosine transversion at nucleotide 920 which replaces the wild type asparagine at codon 307 with a threonine (p.N307T). The third, also a missense mutation due to a thymine to cytosine transition, is located at nucleotide 2422 in exon 14, and replaces the wild-type cysteine at codon 808 with an arginine (p.C808R). The latter mutation detected by SSCP was a previously reported GGCC duplication in exon 8 (c.1186_1187insGGCC, p.R396fsX472; Abramowicz et al., 1992). The duplication introduces a Nae I restriction site that allows rapid identification of the mutation. This mutation was also found in two patients. We ruled out the possibility that the p.N307T and p.C808R mutations could be polymorphisms since they were not detected in 100 chromosomes from the general population by SSCP analysis.

As the majority of the reported mutations occur in exons 8, 9, 10, 12 and 14 (Bakker et al., 2000), we extended our initial screening for TPO mutations by sequencing directly the PCR fragments that contained these exons, in selected patients. Two additional missense mutations were identified (Table 2). One is a guanine to adenine transition at position 1297 in exon 8, which resulted in the substitution of valine for methionine at position 433

(p.V433M). In the other missense mutation detected, the wild-type cytosine at position 1496 was replaced by a thymine, resulting in the replacement of proline by leucine at position 499 in exon 9 (p.P499L). The mutant nucleotide 1297A creates a new Hsp92 II restriction site that is absent in the presence of the wild-type 1297G. Digestion of the mutant allele results in two fragments of 323 and 129 bp. In contrast, the mutant nucleotide 1496T destroys a wild-type Aci I recognition site. The 425 bp fragment amplified from the DNA segment flanking exon 9 of the TPO gene produces fragments of 7, 25, 36, 76, 119 and 152 bp when digested with Aci I, whereas the mutant allele yields fragments of 7, 36, 76, 144 and 152 bp. We made use of Hsp92 II and Aci I restriction analysis to detect these substitutions in the patients and the general population. None of the 100 normal alleles analyzed had the mutated nucleotides 1297A or 1496T, indicating that p.V433M and p.P499L are not polymorphisms. In addition, SSCP and sequencing analysis of the TPO gene revealed several polymorphisms previously described.

Table 2. Mutations in the Human TPO Gene Identified in this Study

Exon	Nucleotide Change [a]	Consequence at protein level	No. of patients	Perchlorate [c] discharge test (%)
5	c.387delC	Frameshift mutation p.N129fsX208	1	80
8	c.920A>C (AAC>ACC)	Missense mutation (p.N307T)	2	69/80
8	c.1186_1187insGGCC	Frameshift mutation [b] p.R396fsX472	2	84/99
8	c.1297G>A (GTG>ATG)	Missense mutation (p.V433M)	1	99
9	c.1496C>T (CCG>CTG)	Missense mutation (p.P499L)	1	99
14	c.2422T>C (TGC>CGC)	Missense mutation (p.C8O8R)	1	77

[a] Position numbering is according to TPO mRNA reference sequences (GenBank Accesion Number: NM_000547). The A of the ATG of the initiator Methionine codon is denoted nucleotide +1. The codon for the initiator Methionine is codon 1. Nomenclature of mutations according to den Dunnen and Antonarakis, 2000.
[b] The mutation was described previously (Abramowicz et al., 1992; Bikker et al., 1995; Bakker et al., 2000).
[c] Release of the intrathyroidal iodide pool, after oral administration of sodium perchlorate at a dose of 1 g.

The eight mutations identified in seven of the 14 patients were all heterozygous. In one patient, a compound heterozygous was found with both mutations located in exon 8 (p.R396fsX472/p.V433M). Five mutations were detected in exon 8 and one in exon 9; exons 8, 9 and 10 encode for the active site of the enzyme since they contain the putative proximal and distal heme binding histidine residues (Medeiros-Neto et al., 1993). As the mode of inheritance of iodide organification defect is autosomal recessive, further sequencing analysis in the rest of TPO exons, or, alternatively, in a regulatory or intronic region is needed in order to identify the additional TPO mutations.

In conclusion, this study shows five inactivating TPO mutations associated with congenital goiter hypothyroidism that have not been described yet: one frameshift mutation (p.N129fsX208) and four missense mutations (p.N307T, p.V433M, p.P499L p.C808R). This report further documents 1) that TPO mutations display significant allelic heterogenity, 2) that the analysis can not be limited to a few exons, and 3) that mutations are likely to occur in regulatory or non-coding regions.

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