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ORIGINAL ARTICLE

Congenital goitrous hypothyroidism: mutation analysis in the thyroid peroxidase gene

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Summary

Background Iodide organification defect (IOD) is characterized by a reduced ability of the thyroid gland to retain iodide resulting in hypothyroidism. Mutations in thyroid peroxidase (TPO) gene appear to be the most common cause of IOD and are commonly inherited in an autosomal recessive fashion. The TPO gene is located on the chromosome 2p25. It comprises 17 exons, covers approximately 150 kb of genomic DNA and codes 933 amino acids.

Objetives In this study, we characterize the clinical and molecular basis of seven patients from four unrelated families with congenital hypothyroidism (CH) because of IOD.

Design and Methods All patients underwent clinical, biochemical and imaging evaluation. The promoter and the complete coding regions of the human TPO along with the flanking intronic regions were analysed by single-strand conformation polymorphism analysis and direct DNA sequencing. Segregation analysis of mutations was carried out, and the effect of the novel missense identified mutations was investigated by 'in silico' studies.

Results All subjects had congenital and persistent primary hypothyroidism. Three novel mutations: c.796C>T [p.Q266X], c.1784G>A [p.R595K] and c.2000G>A [p.G667D] and a previously reported mutation: c.1186_1187insGGCC [p.R396fsX472] have been identified. Four patients were compound heterozygous for p.R396fsX472/p.R595K mutations, two patients were homozygous for p.R595K, and the remaining patient was a compound heterozygous for p.Q266X/p.G667D.

Conclusions Our findings confirm the genetic heterogeneity of TPO defects and the importance of the implementation of molecular studies to determinate the aetiology of the CH with dyshormonogenesis.

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Introduction

Congenital hypothyroidism (CH) is the most frequent endocrine disease in infants, with a prevalence of 1:2000–1:4000 newborns. Primary newborn screening for CH has been adopted by most countries around the world, using either primary T_4 follow-up TSH or primary TSH testing.[1,2] In Argentine, this screening programme was incorporated in 1985 using the TSH determination from a small quantity of blood in special filter paper cards.[2] Thyroid dyshormonogenesis or defects in thyroid hormone biosynthesis, which accounts for 15–20% of the primary CH cases, has been linked to mutations in the sodium iodide symporter (NIS),[3] SLC26A4 (which encodes pendrin, a multifunctional anion exchanger),[4] thyroid peroxidase (TPO),[5] dual oxidase 2 (DUOX2),[6] DUOX maturation factor 1 (DUOXA1),[7] DUOX maturation factor 2 (DUOXA2),[6] dehalogenase 1 (DEHAL1)[8] and thyroglobulin (TG)[9] genes. Song *et al.*[10] have proposed recently that DUOX and TPO proteins could interact to increase the working efficiency and minimize the H2O2 spillage. Defect in such mechanisms could explain some CHs.

Elevated TG serum levels, an increased and rapid uptake of iodine and a significant discharge of thyroidal radioiodine after perchlorate discharge test (PDT) are consistent with iodide organification defect (IOD). Mutations in the TPO gene, DUOX system and SLC26A4 genes are associated with IOD. The latter case is characterized by additional sensorineural hearing loss.

Mutations in TPO appear to be the most common cause of dyshormonogenesis with permanent CH.[5,11,12] Up to now, more than 60 mutations have been described in the human TPO gene, which are commonly inherited in an autosomal recessive fashion. Only about 20% of the cases exhibit only monoallelic defects of TPO, presumably because of unidentified mutations in unexamined

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intronic or regulatory regions of the gene. So, a case of monoallelic expression of only the mutant allele has been reported.[13] The TPO gene is located on the chromosome 2p25. It comprises 17 exons, covers approximately 150 kb of genomic DNA and codes 933 amino acids.[14] Mutations have been classified as missense, nonsense, frameshift and splicing mutations and gene deletions.[5,15–22]

In our study, we present the results of the analyses of the phenotype and the TPO gene mutation in seven patients from four unrelated families with CH and IOD. Three novel mutations: c.796C>T [p.Q266X], c.1784G>A [p.R595K] and c.2000G>A [p.G667D] and a previously reported mutation: c.1186_1187insGGCC/c.1784G>A [p.R396fsX472/p.R595K] have been identified. Four patients were compound heterozygous for p.R396fsX472 and p.R595K mutations, two patients were homozygous for p.R595K mutation, and the remaining patient was a compound heterozygous for p.G667D and p.Q266X. In addition, using a three-dimensional molecular TPO model, a putative pathogenic mechanism of each novel missense identified mutation was evaluated in silico. Our analysis revealed disturbance in the electrostatic surface and alteration of specific amino acid interactions.

Materials and methods

Patients

Patients with CH, goitre, high TG levels and negative anti-TG and anti-TPO auto antibodies were selected to participate in this study. Patients A, B, C, D, E and F were followed at Endocrine Unit of 'Hospital de Niños Santísima Trinidad', and patient G was followed at Endocrine Division of 'Hospital de Niños Ricardo Gutiérrez'. Replacement treatment was started with a daily dose of L-thyroxine (L-T_4) of 12–15 μg/kg of weight for each child. Written informed consent was obtained from the parents of the children involved in this study, and the research project was approved by the institutional review board.

Family 1

Patient A (II-I). The fifth child born from nonconsanguineous parents was presented for the first time at the age of 84 days with clinical manifestation of hypothyroidism. The laboratory tests are shown in Table 1. The ultrasound confirmed diffuse goitre (4 ml). Thyroid volume was estimated by multiplication of length, breadth and depth and a corrective factor (0·52) for each lobe.[23] The combined volume (ml) in newborn infants is 1·62 (SD: 0·41), range: 0·7–3·3.[23] The knee X-ray did not show ossification centres. He started with L-thyroxine (L-T_4) treatment immediately after diagnosis. Height growth was adequate but with a global developmental delay that required psycho-pedagogical assistance and adapted school programme. Goitre persisted even under hormone replacement treatment, and a total thyroidectomy was decided at the age of 16 years. Histological diagnosis was consistent with colloid nodular goitre.

Patient B (II-2). He was born of uneventful pregnancy and delivery at 36 weeks of gestation, with adequate weight and size. His hypothyroidism was detected by high TSH levels (335 mIU/l) on neonatal screening carried out in other centre at age of 6 days. At 36 days of age, the patient was referred to the endocrine centre cited previously. He was re-evaluated (Table 1) and treated with L-T_4 replacement. The ultrasound showed diffuse enlargement of the gland (3·6 ml). The knee X-ray did not show ossification centres. Psycho-pedagogical intervention and a special school programme were required.

Family 2

Patient C (II-1). The patient was the first child of an unrelated couple. She was diagnosed at 27 days of life by newborn screening (TSH: 292 mIU/l), and L-T_4 treatment was started immediately. Eleven month latter, she was derivated to our endocrinology centre. The ^{99}Tc scintigraphy confirmed an orthotopic thyroid gland of normal uptake. She has three healthy siblings and an affected younger brother (patient D:II-2). Control was irregular and treatment compliance was poor. Goitre advanced into grade III–IV. The patient shows learning difficulties. At 12 years of age, the ultrasound showed hyperplasic thyroid gland with a heterogeneous multimicronodular pattern. The patient shows learning difficulties.

Patient D (II-2). This patient was diagnosed by newborn screening on day 4 of life being TSH:151 mIU/l. At the age of 30 days, the diagnosis was confirmed (Table 1). Thyroid ultrasound showed a diffuse goitre (4·4 ml). Treatment with L-T_4 was initiated. There was absence of ossification centres in knee X-rays. The patient did not comply with the treatment adequately. At present, he attends primary school and has learning difficulties.

Family 3

Patient E (III-1). The third child of nonconsanguineous parents was referred at the age of 2 months to Endocrinology unit with symptoms of hypothyroidism and diffuse goitre. Thyroid profile confirmed hypothyroidism with high TG (Table 1) and the presence of goitre (3·6 ml). He began treatment with thyroid hormone replacement immediately. He had a sister with CH, who died at the age of one. The cardiovascular examination showed pulmonary stenosis. He has not complied with treatment adequately. He presented psychomotor and language delay. He needed psycho-pedagogical intervention and a special school programme.

Patient F (III-2). He is the fifth child who was diagnosed by newborn screening at the age of 7 days being the TSH: 390 mIU/l. The laboratory tests are shown in Table 1. Thyroid ultrasound showed an enlarged, homogeneous gland (4·7 ml). He did not presented femorotibial ossification centres. Compliance to treatment was inadequate and needed psychomotor, phonoaudiologic and psycho-pedagogical assistance. He attends primary school and has learning difficulties.

Family 4

Patient G (II-2). The only child of nonconsanguineous parents was born at term with appropriate weight for gestational age.

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Table 1. Laboratory data in patients with congenital goitrous hypothyroidism and organification defect

Families	Patients	Current age (years)	Gender	Age at diagnosis and treatment	Serum TSH (mIU/l)	Serum FT_4 (pmol/l)	Serum TT_4 (nmol/l)	Serum TT_3 (nmol/l)	Serum TG (pmol/l)
1	A	17	M	84 days	>50	0·25	5·41	0·48	916·6
1	B	9	M	30 days	>100	0·64	5·41	0·37	1515
2	C	15	F	27 days	*292	N/A	N/A	N/A	N/A
2	D	13	M	30 days	350	0·13	5·41	0·52	5302·5
3	E	11	M	60 days	420	0·13	5·41	0·54	1908·9
3	F	8	M	7 days	524	1·8	18·02	0·76	496·9
4	G	17	F	21 days	>100	N/A	<12·87	<0·31	N/A
Reference range					0·44–8·8	11·58–29·6	92·68–218·82	1·61–4·04	9·76–262·1

F, female; M, male; N/A, data not available; TG, thyroglobulin.
The values reflect the hormonal situation before L-T_4 substitution.
*292 mIU/l corresponds to the Neonatal TSH. That was the only available hormone determination before the L-T_4 replacement.

When severely depressed (Apgar 3–7), the patient required oxygen. Hypothyroidism was diagnosed at 21 days of life by newborn screening. L-T_4 treatment was initiated immediately. The ^{99}Tc scintigraphy confirmed the presence of a small goitre. There was absence of ossification centres in knee X-rays. In this patient, the treatment was interrupted for a month before re-evaluated thyroid parameters (Table 1) and at this point, PDT of 80%, confirmed the absence of organification of iodide. She grew and developed normally. She received a standard education.

Laboratory testing

Serum total T_4 (TT_4) and serum TSH levels were determined by ECLIA ELECSYSTM system (Roche, Mannheim, Germany). Serum TG concentration was measured using IFMA Delfia (Perkin Elmer, Turku, Finland). Anti-TPO and anti-TG antibodies were determined by ICMA Immulite (Diagnostic Products Corporation, Los Angeles, CA, USA).

PCR amplification

Genomic DNA was isolated from peripheral blood leucocytes by the standard cetyl trimethyl ammonium bromide (CTAB) method.[24] The promoter region and all 17 exons of the human TPO gene including the intronic flanking regions from the affected patients were amplified using the primers and PCR conditions reported previously.[25]

Single-strand conformation polymorphism (SSCP) analysis

Single-strand conformation polymorphism was used to screen for the presence of mutations in each exon of the TPO gene and their flanking intronic regions. The gel matrix for SSCP contained 8% or 10% polyacrylamide (29:1) (Invitrogen, Life Technologies), with or without 10% glycerol. Samples were electrophoresed for up to 18 h at a constant temperature (4 °C). DNA was visualized by silver staining.

DNA sequencing

Samples showing an aberrant pattern in SSCP analysis were directly sequenced using sense- and antisense-specific primers reported previously[25] with the Big Dyedeoxyterminator Cycle Sequencing Kit (Applied Biosystems, Weiterstadt, Germany). The samples were analysed on the ABI Prism 3100 DNA sequencer (Applied Biosystems). The mutation nomenclature refers to the National Center for Biotechnology Information (NCBI) human TPO reference nucleotide sequence (NCBI accession number NM_000547) and is expressed following the standard proposed by the Association for Molecular Pathology Training and Education Committee[26] with the A of the ATG start codon denoted as +1 and the initiator methionine being codon 1.

Identification of c.1186_1187insGGCC mutation by NaeI restriction analysis

The c.1186_1187insGGCC mutation generates a NaeI recognition site.[16] A 460-bp fragment corresponding to exon 8 was generated by PCR[25] and digested with the restriction enzyme NaeI as recommended by the manufacturer (New England BioLabs Inc., Beverly, MA, USA). Then, DNA fragments were electrophoresed in 3% agarose gel stained with ethidium bromide.

Validation of c.1784G>A and c.2000G>A mutations by SSCP analysis

We validated the c.1784G>A and c.2000G>A mutations studying healthy unrelated individuals by SSCP using intronic primers previously reported to amplify exon 11.[25] The gel matrix for SSCP analysis contained 10% polyacrylamide (29:1) without glycerol. Samples were electrophoresed.

Protein homology analysis

Amino acid sequence homology between several TPO species were compared using the MegAlign (DNASTAR, Hauser University of

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California-SF) and Search for Conserved Domains (http://www.ncbi.nlm.nih.gov/BLAST) software programs. The amino acid sequences are based on the GenBank database: *Homo sapiens* TPO (AAA61217.2); *Canis lupus familiaris* TPO (AAM26737.1); *Rattus norvegicus* TPO (EDM03234.1); *Mus musculus* TPO (EDL36934.1); *Culex quinquefasciatus* TPO (XP_001866942.1); *Sus scrofa* TPO (NP_001171681.1).

In silico studies of the identified TPO gene mutations

To approach the three-dimensional structure of the p.G666D and p.R595K TPOs, we modelled the wild-type and mutant TPO variants in silico by using the Swiss Model program package.[27,28] Multiple templates were used to generate the different target models, but the one presenting the best sequence identity percentage was chosen. So, a crystal structure of human myeloperoxidase isoform C (PDB accession no. 1mhl, 2·25- Å resolution) was used to model the modified sites in the two named TPO variants (compared with the wild-type TPO). Myeloperoxidase showed an overall identity of 46·4% for amino acids 257–735 of TPO. Our model shows a good quality according to the 'ProQ' algorithm.[29] We submit the chosen model to 'Swiss PDB Viewer' (spdbv)[30] to determine potential structural and energetic differences between mutants and the wild-type TPO. The surface electrostatic potential of mutants and wild-type structures were computed by solving the Poisson–Boltzmann equation implemented in the program DelPhi.[31] This method has been shown to reliably predict electrostatic potential energies. The secondary structure picture was produced with the The PyMOL Molecular Graphics System (2010) (http://www.pymol.org).

Results

DNA sequence analysis of the TPO gene

The most frequent mutation in TPO gene, the known insertion of 4 bp GGCC in position 1186 of the cDNA (c.1186-1187insGGCC; p.R396fsX472),[16] produces a stop codon in exon 9 and introduces a NaeI restriction site that allows the rapid identification of the mutation. To determinate this mutation, exon 8 of seven patients from four unrelated families was PCR amplified and digested with NaeI. All 17 exons of the TPO gene, along with the flanking intronic sequences, were further screened by SSCP and direct DNA sequencing. Sequence analyses demonstrated that each child had biallelic mutations. Four different TPO mutations were identified, three of them are novel (c.1784G>A [p.R595K], c.796C>T [p.Q266X] and c.2000G>A [p.G667D]) and one is a previously reported mutation (c.1186_1187insGGCC [p.R396fsX472]).

Segregation analysis of the mutations in TPO gene

In family 1, patients A (II-1) and B (II-2) were compound heterozygous. Analyses by NaeI restriction showed that patients A (II-1) and B (II-2), their mother (I-1), brothers (II-3; II-4) and one sister (II-6) presented the c.1186-1187insGGCC [p.R396fsX472] mutation. Therefore, the wild-type allele, which is resistant to digestion by NaeI (fragment of 460 bp) and the mutated allele, which is digested by NaeI (two fragment of 246 and 214 bp) can be observed (data not shown). In addition, patients A (II-1) and B (II-2) and two of their sisters (II-5; II-7) carry a novel missense mutation because of a guanine to adenine transition, located at nucleotide 1784 in exon 11 (c.1784G>A), which replaces the wild-type arginine at codon 595 with a lysine [p.R595K] (Fig. 1). This mutation was detected by SSCP analysis. Father of family 1 was not available for segregation analysis. Two known different polymorphisms are located around the c.1186_1187insGGCC mutation in patients A and B, p.A373S and p.S398T both of them being homozygous for serine 373 and heterozygous for threonine 398.

In family 2, we have detected the mutations described previously, c.1186-1187insGGCC [p.R396fsX472] and c.1784G>A [p.R595K] respectively in both patients (C and D) whom inherited a copy of c.1186-1187insGGCC mutation from their mother and a copy of c.1784G>A transition from their father (Fig. 2).

In family 3, patients E and F (III-1 and III-2) carried the novel mutation c.1784G>A in exon 11 in a homozygous form, which leads to the replacement of arginine at position 595 with a lysine. The patients' parents (II-1 and II-2) and one brother (III-3) resulted heterozygous carriers of p.R595K (Fig. 3). The other brother presents the wild-type alleles for c.784G>A. An abnormal pattern of migration was detected by PCR-SSCP from patients E and F. Molecular diagnosis confirmed the presence of CH and goitre in the patients' aunt, who presented all the features of this pathology. She was homozygous for p.R595K mutation (Fig. 3).

In family 4, two novel mutations have been identified in patient G by SSCP analysis. One of them was inherited from the father and consists of a transition of guanine to adenine at nucleotide 2000 in exon 7 (c.2000G>A), which replaces the wild-type glycine at codon 667 with aspartic [p.G667D]. The other mutation was a nonsense mutation in exon 11 of the TPO gene (c.796C>T) changing the glutamic at position 266 by a stop codon [p.Q266X] (Fig. 4).

Validation of the c.1784G>A and c.2000G>A mutations by SSCP analysis

We ruled out the possibility that the c.1784G>A and c.2000G>A mutations could be polymorphisms as these were not detected in 103 chromosomes from the general population by SSCP analysis.

Protein homology analysis

The deleterious effect of the p.R595K and p.G667D missense mutations was therefore evaluated by assessing the degree of evolutionary conservation of the respective amino acids, among human and other animal wild-type peroxidases. Multiple sequence alignment of the human TPO with sequences found in the GenBank database, using Clustal method, revealed that wild-type arginine residue at position 595 and that wild-type glycine at position 667 are strictly conserved in all TPO species analysed.

In silico studies of the identified TPO gene mutations

In silico studies were performed to elucidate a correlation between structural disturbances and putative functional commitment,

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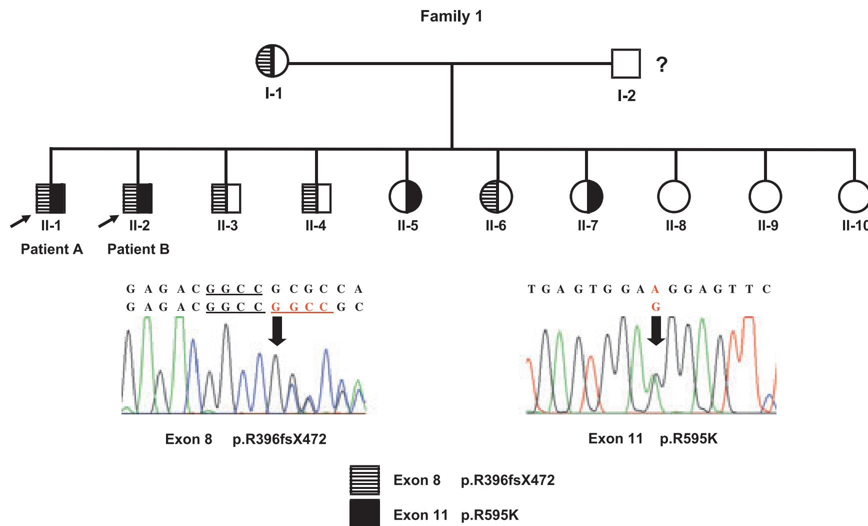

Fig. 1 Segregation analyses of the thyroid peroxidase-mutated alleles in members of the family 1. Automated fluorescence-based sequencing chromatograms of exons 8 and 11 from patients II-1 and II-2. Arrows show the nucleotide change for c.1186_1187insGGCC [p.R396fsX472] and c.1784G>A [p.R595K] mutations.

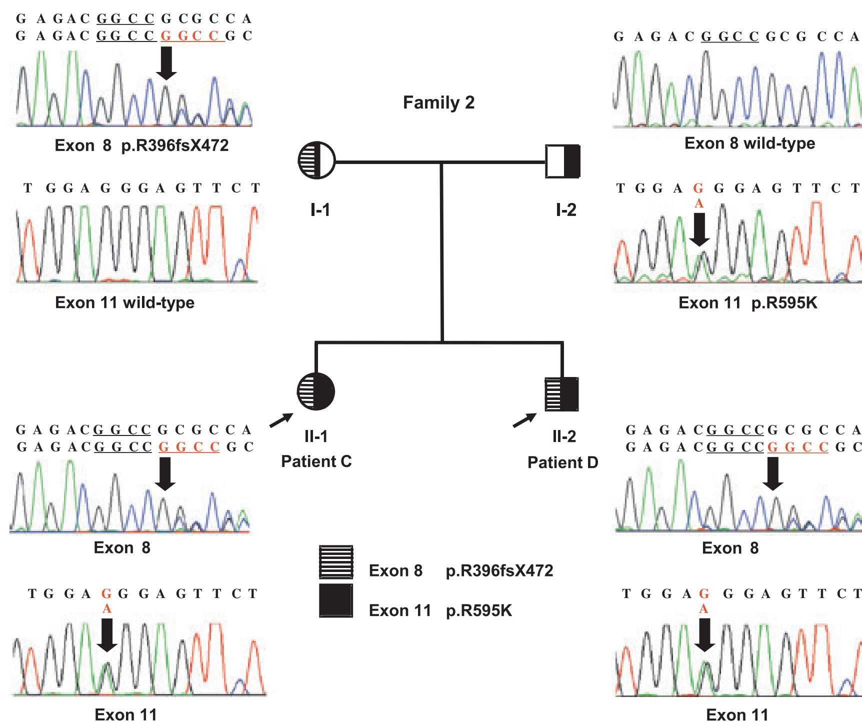

Fig. 2 Segregation analyses of the thyroid peroxidase-mutated alleles in members of the family 2. Automated fluorescence-based sequencing chromatograms of exons 8 and exon 11. Arrows show the nucleotide change for c.1186_1187insGGCC [p.R396fsX472] and c.1784G>A [p.R595K] mutations.

achieving a possible explanation of the pathogenic mechanism of the novel missense mutations analysed. Both p.G667D and p.R595K are located within highly conserved region across species, which suggests an important role in the function and/or structure of TPO. To evaluate the three-dimensional impact of these mutations, we performed an homology model of TPO to compute and compare mutants and wild-type structures. We considered both identity percentage between MPO and TPO (46·4% in the modelled residue range between 257 and 735 of TPO) and a good quality assessment with the ProQ algorithm (Predicted LGscore: 3·742, Predicted MaxSub: 0·372), as good indicators of reliability of the model proposed (Fig. 5a). One of the mentioned mutations, p.G667D, showed the generation of a new favourable hydrogen bond and a disruptive interaction between the aspartic acid in

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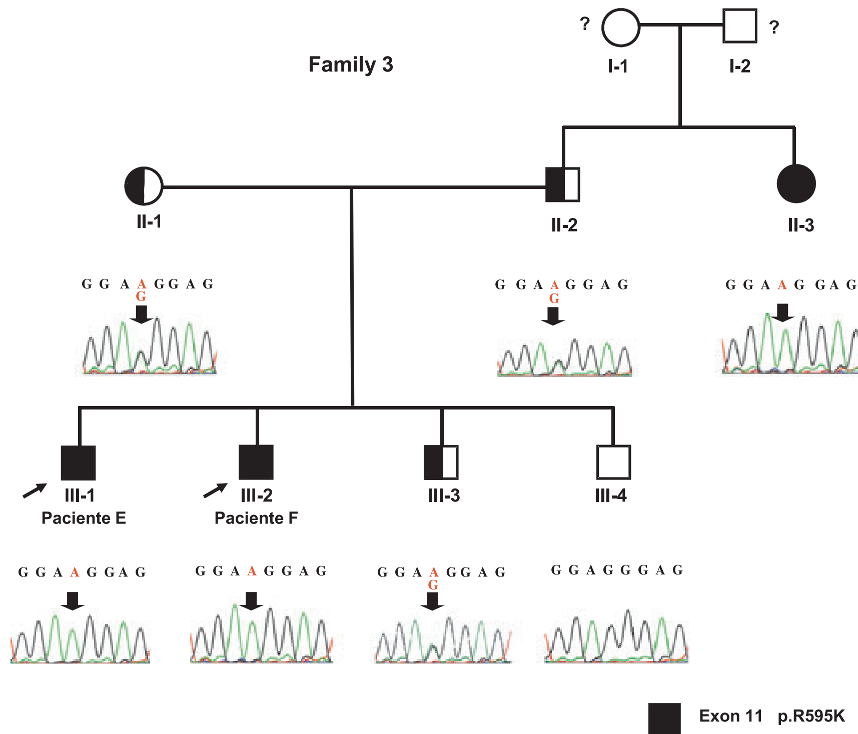

Fig. 3 Segregation analyses of the thyroid peroxidase-mutated alleles in members of the family 3. Automated fluorescence-based sequencing chromatograms of exon 11 of patients III-1 and III-2. Arrows show the nucleotide change for c.1784G>A [p.R595K] mutation. PCR-SSCP analysis of exon 11. II-1, II-2: patients; N1, N2, N3: Control individuals.

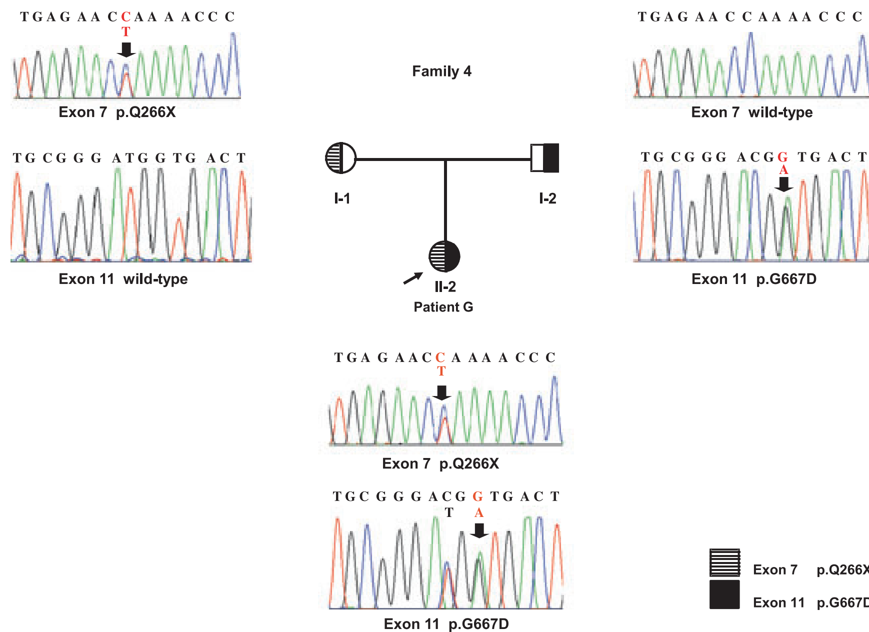

Fig. 4 Segregation analyses of the thyroid peroxidase-mutated alleles in members of the family 4. Automated fluorescence-based sequencing chromatograms from family 4 members. Arrows show the nucleotide change for c.796C>T [p.Q266X] in exon 7 and c.2000G>A [p.G667D] mutations.

position 667 and a proline located in 479 position. In addition, an electrostatic surface alteration is produced resulting in a more electronegative region not only around the mutation but also far away in the molecular structure (Fig. 5b). By contrast, the other substitution, p.R595K, breaks two hydrogen bonds with a proline in position 601 and another hydrogen bond with a glutamic acid in position 641, generating a new hydrogen bond interaction with a tyrosine in position 591. In addition, an electrostatic potential

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change of the molecule arises replacing a net electropositive area by a more neutral surface (Fig. 5c). The results obtained for the predicted minimization energy changes after theses amino acids substitutions define not only a local but a global unfavourable structural condition, denoting a considerable energetic cost in global three-dimensional structure stabilization (TPO WT: global minimization energy = −23 043·94 kJ/mol, p.G667D: global minimization energy = −21 287 383 kJ/mol, p.R595K: global minimization energy = −22746 428 kJ/mol). Computations were performed in vacuo with the GROMOS96 43B1, without reaction field.[32]

Discussion

Here, we demonstrate seven children with CH because of biallelic inactivating mutations in the TPO gene. Molecular analyses indicate that patients A, B, C and D are compound heterozygous for p.R396fsX472 and p.R595K mutations, patients E and F are homozygous for p.R595K mutation, and patient G is a compound heterozygous for p.G667D and p.Q266X mutations.

Patients with IOD have a variable degree of primary hypothyroidism and thyroid gland enlargement depending on the severity of the defect. In untreated patients, a complete defect causes a severe phenotype resulting in mental retardation with a large goitre. Unfortunately, this is the case of 6 of our patients (A, B, C, D, E and F) who needed psycho-pedagogical, phonoaudiological and/or psychomotor assistance. Only one patient (G) grew and developed normally.

Perchlorate discharge test and the measurement of TG serum concentration help to differentiate patients with iodide organification disorder of those with DEHAL1 or TG deficiencies. In patients with congenital goitre, very low TG concentrations are specific for the diagnosis of TG defects. Whereas patients with IOD or DEHAL1 mutations have elevated or very elevated serum TG levels. As mutations in the TPO gene are the most likely cause of dyshormonogenetic congenital goitrous hypothyroidism because of permanent IOD,[5] we analysed the TPO gene of all affected individuals.[33] When PDT is not available, screening for TPO mutations is still reasonable.[34] Unfortunately, PDT from patients A, B, C, D, E and F was unavailable. In the present study, a discharge of 80% indicated a severe defect in patient G.

Thyroid peroxidase is a membrane-bound glycoprotein located at the apical membrane of the thyroid follicular cells that catalyses both iodination and coupling of iodotyrosine residues within the TG molecule, leading to the synthesis of thyroid hormone. The preprotein is composed of a putative 14-amino acid signal peptide followed by a 919-amino acid polypeptide that involves a large extracellular domain, a transmembrane domain and a short intracellular tail.[35] The amino acid sequences between 149 and 711, corresponding to exons 5–12 in the human TPO gene, show some significant similarities with animal haem peroxidase (An peroxidase) consensus (Fig. 5). Based on the literature, exons 8, 9 and 10 encode the catalytic centre of the TPO protein (haeme-binding region), which is crucial for the enzymatic activity. The next two exons, 13 and 14, belong to the complement control protein (CCP)-like (residues 742–795) and calcium-binding epidermal growth factor (EGF)-like (residues 796–839) gene families, respectively[35] (Fig. 5). Exon 15 codes for the transmembrane part of the protein and exons 16 and 17 for its cytoplasmic tail. The truncated

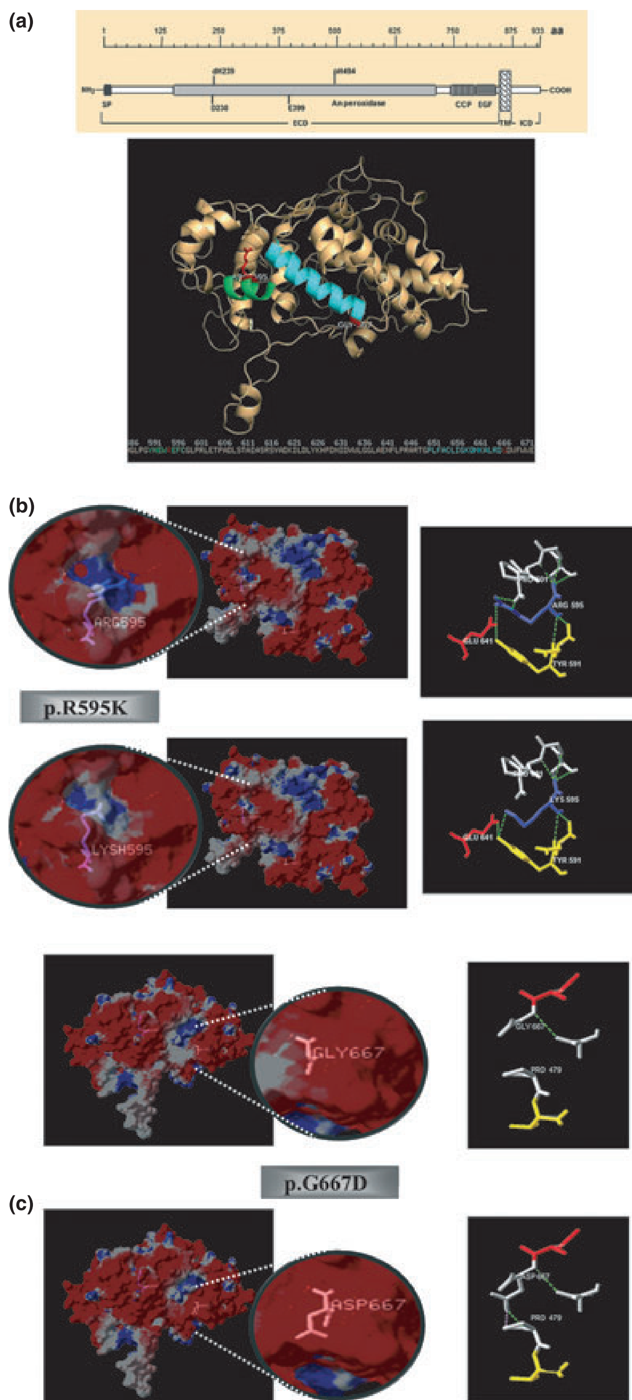

Fig. 5 Structural analysis. (a) Top panel: Schematic representation of the domains in the wild-type human thyroid peroxidase protein. Bottom panel: Ribbon representation of the modelled structure of thyroid peroxidase. Residues implicated in the novel missense mutations identified (G666 and R595) are indicated. (b) Surface electrostatics of the wild-type R595 and mutant K595. Acidic regions are depicted in red and basic ones in blue. (c) Surface electrostatics of the wild-type G667 and mutant D595. Acidic regions are depicted in red and basic ones in blue.

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protein of 265 amino acids, generated by the novel c.796C>T mutation in exon 7 [p.Q266X], causes the loss of the catalytic part of the enzyme. So, great part of one of the main functional domains of the TPO such as the An peroxidase is eliminated. The CCP-like and EGF-like, transmembrane and intracytoplasmatic domains are also missing. The functional consequences of the truncated protein are a complete loss of TPO activity. The p.R595K and p.G667D missense mutations are located in the An peroxidase domain of TPO gene. Residues at positions 595 and 667 are conserved in all species for which suitable TPO sequences have been reported. Using the molecular TPO model proposed, we could predict some structural and energetic commitments that could explain functional impairment in patients presenting the mentioned mutations. In particular, the change of a basic arginine in position 595 by a basic lysine, *a priori*, a net change in the surface charge of the protein would not be expected. However, according to our in silico analysis, we could determinate that the alteration of amino acids interactions and a large electrostatic environment disturbance on the molecular surface could have consequences on the redox potential of the mutant TPO, which might impair proper iodide oxidation. This hypothesis could explain why the function is committed and patients who carry the p.R595K mutation in a homozygous form presented severe phenotype (Patients E and F). Otherwise, we have identified this mutation in two unrelated families who also carried the p.R396fsX47 mutation. With respect to other novel identified mutation, the p.G667D consists in the replacement of a neutral nonpolar residue such as glycine by an aspartic acid at position 667, which is expected to affect amino acids interactions between closer residues of the protein. Accordingly, the consequent electrostatic disturbance could impair the TPO biological activity. This is consistent with a functional study of the nearby mutation p.R665W, which was detected in a patient with severe CH.[36] The mutation is shown to disturb plasma membrane localization in the mutated TPO protein. Glycine 667 in TPO forms part of an α helix that interconnects two locally folded regions.[37]

In summary, we identified three novel mutations (c.1784G>A [p.R595K], c.796C>T [p.Q266X] and c.2000G>A [p.G667D]) and one previously reported (c.1186_1187insGGCC [p.R396fsX47]) in thyroid peroxidase gene responsible for severe phenotypes of iodide organification defects. The homology modelling was a useful approach to study changes on the structure and molecular surface of thyroid peroxidase to explain functional impairment and the consequent clinical manifestations found in our patients. The exact molecular diagnosis avoids the negative effects associated with delayed diagnosis and treatment of hypothyroidism on mental development and enables the early identification of successive cases in the same family.

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