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ORIGINAL ARTICLE

Analysis of *TPO* gene in Turkish children with iodide organification defect: identification of a novel mutation

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Abstract The objective was to determine molecular genetic analysis of the *TPO* gene in Turkish children with iodide organification defect (IOD). Patients with a diagnosis of primary hypothyroidism were evaluated. Subjects having a definite diagnosis of autoimmune thyroiditis, thyroid gland dysplasia and, or iodine deficiency were excluded. A total of 10 patients from nine unrelated Turkish families, with an unknown etiology of hypothyroidism, and with a presumptive diagnosis of IOD were included in the study. A perchlorate discharge test (PDT) was performed to all subjects, and sequence analysis of *TPO* gene was applied in patients with a positive PDT. Five out of 10 patients have a total IOD, while the five remaining patients have a partial IOD according to PDT results. In two sisters, one has a partial and the other one has a total IOD a novel homozygous nonsense p.Q315X mutation was found in exon 8. Additionally, a previously known homozygous missense p.R314W mutation was detected in the same exon in another patient with a total IOD. No TPO gene mutation was detected in any of the seven remaining patients. Two different *TPO* gene mutations were found to be responsible for IOD in two unrelated Turkish families from the same ethnic background. More subjects should be screened for detecting the prevalence and spectrum profile of *TPO* mutations in our population that might be helpful for understanding the pathophysiology of congenital hypothyroidism.

Keywords Hypothyroidism · *TPO* gene · Perchlorate discharge test

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Introduction

Hereditary inborn errors of thyroid hormone synthesis or thyroid dyshormonogenesis account for 10–15% of congenital hypothyroidism. Different defects in thyroid hormone biosynthesis have been identified so far. Among the pathologies resulted in dyshormonogenesis, thyroid peroxidase (TPO) deficiency caused by *TPO* gene mutations is the most common enzymatic defect responsible for over 50% of the cases [1]. Mutations of various genes, such as sodium iodide symporter [2] and the thyroglobulin (Tg) [3] genes have also been reported in literature. The *TPO* gene is located on the short arm of chromosome 2 (2p25) and comprises 17 exons, and covers approximately 150 kb of genomic DNA [4]. TPO is a membrane-bound glycoprotein located at the apical membrane of the thyroid follicular cells that catalyzes both iodination and coupling of iodotyrosine residues within the Tg molecule, leading to the synthesis of thyroid hormone.

TPO defects are commonly inherited in an autosomal recessive fashion. Inactivating mutations in the *TPO* gene lead to iodide organification defect (IOD) that can be partial or total depending on the type of the mutation and its location [5]. Total IOD is characterized by a rapid and nearly complete release of accumulated intrathyroidal free radioiodine by the administration of perchlorate ions in radionuclide

uptake studies, or in perchlorate discharge test (PDT). Two other conditions are associated with IOD which can be diagnosed with a positive PDT. One involves dual oxidase 2 (DUOX2) enzyme which generates the hydrogen peroxide (H_2O_2) required for TPO activity. Biallelic inactivating mutations in *DUOX2* gene (MIM 607200) have been described as a cause of total IOD in the literature [6]. The other is Pendred syndrome (MIM 274600) which is caused by mutations in the *PDS* gene, and characterized by sensorineural hearing loss and goiter [7].

In our study, we present the *TPO* gene mutation analysis results in a group of patients from the same ethnic background (Turkish Caucasian) living in the same region of Mediterranean. In two sisters with goiter we found a novel homozygous nonsense mutation (p.Q315X) in exon 8, which has not been described before. Unfortunately, the elder sister had mental retardation and very short stature with a large goiter because of the late diagnosis.

Materials and methods

The study was initiated in our out-patient clinic of Pediatric Endocrinology in August 2005 when the national neonatal screening program for hypothyroidism had not yet been established. Patients aged between 0 and 16 years with a diagnosis of primary hypothyroidism defined as high serum TSH, and low serum free T4 (FT4) levels were evaluated. Serum FT4, TSH, Tg, and urinary iodine excretion were measured at diagnosis, and a thyroid ultrasound was performed. Serum TPO antibody (TPOab) and Tg antibody (TgAb) were obtained if autoimmune thyroiditis was suspected. Patients having a definite diagnosis of autoimmune thyroiditis, thyroid gland dysplasia and, or iodine deficiency were excluded. Subjects with an unknown etiology of primary hypothyroidism, and with a presumptive diagnosis of IOD (children with high TSH and Tg levels, and low FT4 levels with goitrous thyroid gland in sonography) were included in the study. Presenting clinical and laboratory findings of the subjects were recorded. The Local Medical Ethics Committee approved the study, and informed consent was obtained from the parent, or guardian of each participating subject. A PDT was performed to all subjects after L-thyroxine (LT4) treatment was discontinued for 4 weeks. Denaturing high pressure liquid chromatography (DHPLC) screening and then the sequence analysis of *TPO* gene was performed in subjects with a positive PDT.

Assays and laboratory tests

All measurements were performed at our university laboratory setting. Samples of urine and blood were obtained from each participant in the morning after an overnight fast. Serum FT4, TSH, Tg, TPOab, and TgAb levels were measured by electrochemiluminescence immunoassay (ECLIA) kits (Roche Diagnostics, Manheim, Germany). Spot urinary iodine excretion was measured by urinary iodine assay kits (Bioclone, Australia). Iodine deficiency was defined as spot urinary iodine excretion <100 μg/l.

Thyroid ultrasonography was performed by a radiologist using the same equipment. Thyroid volume was estimated by multiplication of thickness, width, length, and a corrective factor (0.52). A goiter was defined as a total thyroid volume >+2 standard deviation score (SDS) according to our age-specific reference data [8].

A PDT was performed after the 4-week LT4-off period. ^{131}I was given orally and thyroidal radioiodine uptake was measured by gamma camera 4 h later, at which time potassium perchlorate ($KClO_4^-$) was given orally (10 mg/kg). One hour later, a second measurement of radioactivity was obtained. A decline of less than 10% in the levels of radioiodine was considered to be normal. Values within 10–50% were defined as partial IOD, while values above 50%, as total IOD [5].

Molecular genetic analysis

Genomic DNA was extracted from peripheral blood samples of the affected subjects according to non-enzymatic method [9]. The 17 exonic regions of the *TPO* gene were amplified by PCR with the primers as described by Bikker et al. [10]. Each amplification reaction mixture contained in a final volume of 25 μl of a master mix (Fermentas), containing 1 μl of genomic DNA (50–250 ng), 1 pmol of each forward and reverse primers, and done in a 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA). All 18 amplicons (exon 8 subdivided into two fragments as 8A and 8B) were applied to DHPLC screening (Transgenomics, CA, USA) after mixing with 1:1 ratio with control amplicons. Amplicons presenting abnormal electrophoretic pattern were sequenced by using Big Dye Terminator kit v3.1 (Applied Biosystems) in ABI 310 Sequencer. Sequence variations were compared with the reference *TPO* gene sequence (NM_000547).

Results

A total of 10 patients (3 male and 7 female) from nine unrelated Turkish families were evaluated in detail. Presenting clinical and laboratory findings of the patients were given in Table 1. Urinary iodine excretion levels were normal in these patients. In nine patients goiter was detected ultrasonographically. Serum TPOab and TgAb levels were found to be normal in patients 1, 3, and 7 in

Table 1 Laboratory and clinical findings of the patients at diagnosis, and the results of the molecular analysis

Patient no.	Age (month)	FT4	TSH	Tg	Thyroid USG	PDT (%)	*TPO* gene polymorphisms	*TPO* gene mutations
1	123	0.62	476	N/A	Goitrous	95	p.A373S p.S398T	Neg.
2	1	0.65	73.2	61.6[a]	Goitrous	27.8	p.D666D	Neg.
3	6	0.20	100	1100	Goitrous	42.6	p.A373S p.S398T p.D666D	Neg.
4	1.5	0.16	110	34.9[a]	Normal[a]	16.1	p.D666D	Neg.
5	3.5	0.16	510	1690	Goitrous	58.6	p.S398T	Neg.
6	1	0.50	80	N/A	Goitrous	95.6	p.A373S p.S398T	p.R314W
7	153	0.06	1588	1000	Goitrous	81.9	p.S398T	p.Q315X
8	1	0.60	191	51.6[a]	Goitrous	32.6	p.A257S p.A373S p.S398T p.D666D	Neg.
9	1	0.05	1200	1220	Goitrous	52.7	p.A257S p.A373S p.S398T	Neg.
10	1	0.28	1000	754	Goitrous	48.6	p.S398T	p.Q315X

[a] Under the treatment of LT4

N/A Not available

FT4: N: 0.93–1.70 ng/dl

TSH: N: 0.27–4.20 μIU/ml

Tg Thyroglobulin, N: 1.4–78 ng/ml

PDT Perchlorate discharge test, N: <%10

whom autoimmune thyroiditis was suspected. Among 10 patients, 5 had total IOD, and 5 had partial IOD according to PDT results (Table 1). Two patients (number 1 and 7) have severe mental retardation due to late diagnosis of hypothyroidism.

In patient 6, a previously described [11] homozygous missense c.1030C>T, p.R314W mutation was found in exon 8 (Fig. 1). Both of the parents and the other two kids were found to be euthyroid both clinically and biochemically. Unaffected consanguineous parents were found to be heterozygous carriers for the same mutation.

In patient 7 who was referred to our clinic due to mental retardation, short stature and a large goiter a novel homozygous nonsense c.1033C>T, p.Q315X mutation was detected in exon 8 which has not been described before (Fig. 2). Further investigations in other members of the family revealed that both of the parents and the other three kids were euthyroid both clinically and biochemically. However, severe hypothyroidism and goiter was detected in the newborn sister (patient 10) at 1 month of age. The patient had the same homozygous nonsense mutation (p.Q315X) in exon 8. The unaffected consanguineous parents were heterozygous carriers for the same mutation (Fig. 3).

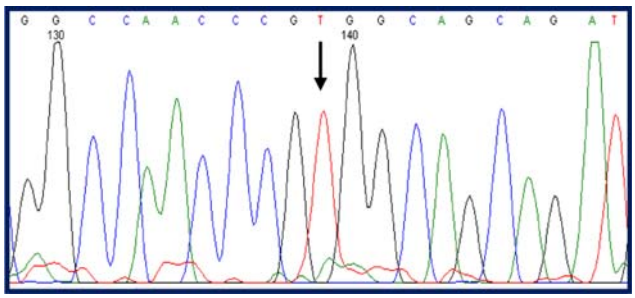

Fig. 1 Sequence electropherogram for part of exon 8 of the *TPO* gene showing the homozygous c.1030C>T, p.R314W mutation in patient 6

The overall frequency of *TPO* mutant alleles in Turkish patients was found as (6/20) 30%. Furthermore, among 10 patients four different previously known [12] *TPO* gene polymorphisms were also detected in the reading frame; c.859G>T, p.A257S (exon 7); c.1207G>T, p.A373S (exon 8); c.1283G>C, p.S398T (exon 8); c.2088C>T, p.D666D (exon11) (Table 1).

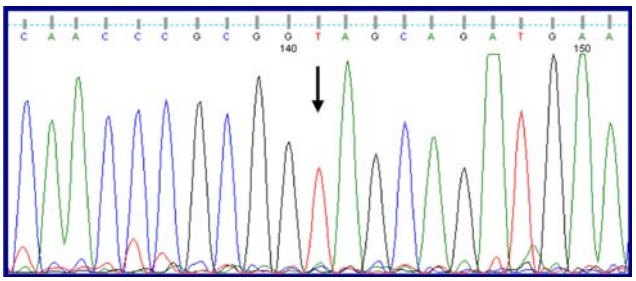

Fig. 2 Sequence electropherogram for part of exon 8 of the *TPO* gene showing the novel homozygous c.1033C>T, p.Q315X mutation in patients 7 and 10

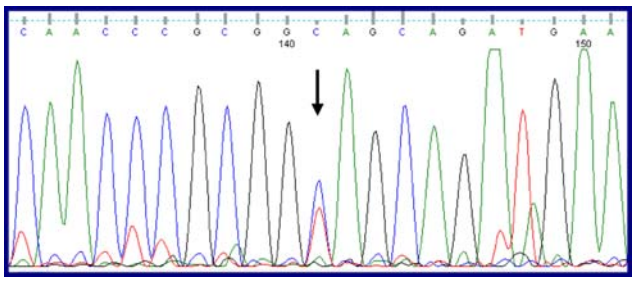

Fig. 3 Sequence electropherogram for part of exon 8 of the *TPO* gene showing the heterozygous c.1033C>T, p.Q315X mutation in the parents of patients 7 and 10

Discussion

Patients with organification defects 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 severe hypothyroidism resulting in mental retardation with a large goiter. In our study, we unfortunately detected two untreated patients (number 1 and 7) with mental retardation, short stature, and goitrous thyroid gland. According to clinical and laboratory findings, patients probably exposed to deleterious effects of thyroid hormone deficiency since their first years of lives. Both patients have nearly complete release of accumulated intrathyroidal free radioiodine by the administration of perchlorate ions in PDT which shows us the severity of the organification defects in thyroid cells.

In the present study, clinical and laboratory findings are quite concordant with the genetic analysis results of the patients 6, 7, and 10. In two sisters (patients 7 and 10) we detected a novel homozygous nonsense mutation in exon 8, and in patient 6 from a different family another homozygous missense mutation was detected in the same exon. Based on the literature, it is reported that exons 8, 9, and 10 encode the catalytic center of the TPO protein (heme-binding region) which is crucial for the enzymatic activity [13]. The TPO enzyme activity depends on both proper folding and membrane insertion, and an intact catalytic site. An amino acid substitution caused by a missense or a nonsense mutation either induces a three-dimensional change or disrupts the glycosylation consensus sequence leading to impaired folding. Thus, the misfolded mutant TPO being trapped in the endoplasmic reticulum, its expression at the apical membrane is impaired [14]. On the other hand, these findings also indicate that these exons harbor mutational hot spots. Thus, mutations in these regions are expected to have major effects on TPO activity resulting in severe organification defect and severe hypothyroidism. This may possibly explain the severe clinical courses of our patients.

Interestingly, in both sisters carrying the same homozygous mutation, the degree of organification defect as evidenced by PDT results was quite different. One had partial IOD, whereas the other had total IOD. However, the presenting clinical findings and the degree of hypothyroidism at diagnosis are very severe in both patients. The difference may be caused by PDT procedures. The inter-individual or intraindividual factors that may affect the outcome of the test, such as variable gastrointestinal absorption rates of perchlorate ions or different amounts of perchlorate ions needed to completely inhibit thyroid iodide transport which both depend mostly on age of the patients, need to be considered as interpreting the test results.

In seven remaining patients with IOD we unexpectedly did not detect any *TPO* gene mutation except some previously known polymorphisms. It can probably be explained by technical limitations of the direct sequencing and haplotype analysis (e.g., small deletions between the polymorphic sites, or mutations in regulatory regions of the *TPO* gene). Additionally, *DUOX2* gene mutations should also be considered as cause of IOD in these patients [15]. On the other hand, 4 out of 7 patients have a partial IOD according to PDT results that can be another reasonable explanation for this situation. In literature, it is clear that total IODs result from homozygous or compound heterozygous mutations in *TPO* gene whereas the etiology of partial IODs is not as clear as in total IODs [16]. It is reported that partial IODs detected in patients with hypothyroidism may be caused by heterozygous or, compound heterozygous mutations of *TPO* gene. However, a mutation cannot always be detected even the two known responsible genes; *DUOX2* and *TPO* have also been screened. The molecular mechanism for the partial IOD is not yet completely understood [5].

In conclusion, we described a novel homozygous nonsense mutation (Q315X) in exon 8 of the *TPO* gene. Additionally, in patients with partial IODs, a mutation in *TPO* gene could not always be detected due to some molecular mechanisms that cannot be identified yet. *DUOX2* gene mutations should also be considered as a

cause. Furthermore, inter- and intraindividual variables in PDT should be taken into account while interpreting the test results.

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