

## CLINICAL STUDY

# High prevalence of thyroid peroxidase gene mutations in patients with thyroid dyshormonogenesis

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## Abstract

**Objective:** Thyroid dyshormonogenesis is a genetically heterogeneous group of inherited disorders in the enzymatic cascade of thyroid hormone synthesis that result in congenital hypothyroidism (CH). Thyroid peroxidase gene (*TPO*) mutations are one of the most common causes of thyroid dyshormonogenesis. The aim of this study was to identify *TPO* gene defects in a cohort of patients with thyroid dyshormonogenesis from Slovenia, Bosnia, and Slovakia.

**Design and methods:** Forty-three patients with permanent CH and orthoptic thyroid glands from 39 unrelated families participated in the study. Mutational analysis of the *TPO* gene and part of its promoter consisted of single-stranded conformation polymorphism analysis, sequencing, and restriction fragment length polymorphism (RFLP) analysis.

**Results:** *TPO* gene mutations were identified in 46% of participants. Seven different mutations were identified, four mutations of these being novel, namely 613C>T (R175X), 1519\_1539del (A477\_N483del), 2089G>A (G667S), and 2669G>A (G860R). Only a single allele mutation was identified in 65% of the *TPO* mutation carriers.

**Conclusions:** The results showed a higher prevalence of *TPO* gene mutations in thyroid dyshormonogenesis when compared with published studies. The high percentage of single allele mutations implied possible intronic or regulatory *TPO* gene mutations or monoallelic expression.

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## Introduction

Congenital hypothyroidism (CH) is the most common congenital endocrine disorder, occurring in 1:3000–4000 newborns (1). The serious detrimental effect on the child's cognitive and motor development, which used to be a major feature of the disease, is now mostly prevented by newborn screening detection and early treatment (2).

In Slovenia, neonatal screening for CH was introduced in 1981 (3). The present incidence of permanent CH in Slovenia is approximately 1:3100 newborns, based on 58 patients identified among 181 264 newborns in a 10-year period from 1994 to 2003 (data obtained from the National Registry of the Department of Endocrinology, Diabetes and Metabolism, University Children's Hospital, Ljubljana, which is the nationwide referral center for CH). The neonatal thyroid-stimulating hormone (TSH) screening program in the Federation of Bosnia and Herzegovina (major part of Bosnia and Herzegovina) has been implemented since March 2005. The Federal Minister of Health accredited the implementation of the neonatal screening program for

CH and phenylketonuria to the Laboratory for the Detection of Congenital Metabolic Diseases at the Department of Pediatrics, University Hospital, Tuzla (4). There were 30 068 newborns screened and 12 patients identified with CH from 1st March 2005 to 31st December 2006 (data obtained from the Federal Registry for CH of the Division for Endocrinology, Diabetes and Metabolism, Department of Pediatrics, University Hospital, Tuzla). The recent population-based studies showed Slovene adolescents and Bosnian schoolchildren to be iodine sufficient (5, 6).

The majority of sporadic CH is due to developmental disorders, referred to as thyroid dysgenesis, which include the complete absence of thyroid tissue (agenesis) and a small-sized thyroid (hypoplasia) with or without associated ectopy. The less common causes of CH are hereditary defects in the enzymatic cascade of thyroid hormone synthesis, which share a common name, thyroid dyshormonogenesis. In this entity, the thyroid gland is found in the normal anatomical position; it is often enlarged, but with adequate levothyroxine (LT<sub>4</sub>) substitution therapy it may also be of normal size. In a 10-year period from 1994 to 2003, 34% of Slovene

patients with permanent CH showed clinical characteristics indicative of thyroid dyshormonogenesis, which is a considerably higher proportion than reported by other groups (2, 7).

Thyroid peroxidase (TPO) enzyme is a thyroid-specific glycosylated hemoprotein with a short transmembrane domain that binds it to the apical membrane of the thyrocyte (2), with the catalytic part facing inside the follicle. It consists of 933 amino acids that are encoded by an mRNA of 3048 nucleotides (8). The *TPO* gene spans over 150 kb on the short arm of chromosome 2, locus 2p25, and consists of 17 exons (9, 10). Published molecular genetic studies suggest that *TPO* gene mutations are one of the most common causes of thyroid dyshormonogenesis, with several different inactivating mutations being identified in patients with total iodide organification defects (TIOD; (7, 11–28)). The inheritance is autosomal recessive (16, 29). *TPO* gene mutations are also infrequently reported in patients with milder thyroid hormone insufficiency or partial iodide organification defects (PIOD; (30, 31)). In the present study, the results of *TPO* gene mutational analysis in 43 patients with thyroid dyshormonogenesis from Slovenia, Bosnia, and Slovakia and their major clinical characteristics are presented.

## Subjects and methods

### Patients

Patients with permanent CH with morphological characteristics of dyshormonogenesis, except for those with low thyroglobulin (Tg) levels, were invited to participate in the study. The diagnosis of CH was based on elevated TSH levels in heel puncture blood samples collected on filter paper between days 3 and 5 of life (cutoff limit 8 mU/l; DELFIA Neonatal hTSH, Perkin-Elmer Life and Analytical Sciences, Wallac Oy, Finland) and confirmation by subsequent determination of TSH, T<sub>4</sub>, tri-iodothyronine, and Tg levels in venous blood between days 7 and 14 of life, when again for TSH the cutoff limit 8 mU/l was used. Patients started replacement therapy with LT<sub>4</sub> within the first 2 weeks of life and continued therapy for at least 2 years. The condition was reevaluated at the age of 2 years, after 4 weeks of therapy withdrawal. Patients with repeatedly elevated TSH above the upper-normal limit 5.50 mU/l (normal values 0.35–5.50 mU/l) were considered to have permanent CH. At the reevaluation at the age of 2 years, thyroid size was evaluated by ultrasound and/or <sup>99</sup>Tc pertechnetate scan. Patients with *in situ* thyroid gland of normal size or enlarged (thyroid volume (32) more than >0.7 cm<sup>3</sup> or thyroid width (33) more than >1.4 cm) were considered to have dyshormonogenesis. Patients with permanent CH and *in situ* normal or enlarged thyroid gland, but with

lower than normal Tg level (normal values 2–70 ng/ml) were not invited to participate. The perchlorate discharge test was not performed; therefore, patients with iodide organification defects were not preselected. The study protocol was approved by the local ethics committees.

A total of 43 patients from 39 apparently unrelated families consented to participate in the study. At the time of genetic analysis, the mean age of participants was 11.07 years (range 2–23 years). Written informed consent was obtained from the participants and parents in the case of minors. Thirty patients from 27 families were Slovene, 11 patients from 10 families were Bosnian, and two unrelated patients were Slovak. Six participants had neonatal total or free T<sub>4</sub> within the normal range and serum TSH level above the cutoff but <20 mU/l. However, all of these patients required permanent replacement therapy, as confirmed at the age of 2 years.

### DNA isolation and amplification

Genomic DNA was isolated from peripheral blood using the salting out procedure in all participants. Each of the 17 exons of the *TPO* gene, including the splicing regions, was amplified by PCR. Primers and amplification conditions are available upon request from the corresponding author. Exon 8 was amplified using two pairs of primers, due to its length. Fifty microliters of PCR mixture were composed of 1 × Gold PCR buffer, MgCl<sub>2</sub>, 200 μM each deoxyNTP, 0.4 μM each primer, 0.4 U AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA), and 200 ng DNA. Five percent of dimethyl sulfoxide were added for amplification of exon 8b. Thermal cycling conditions were as follows: initial denaturation at 95 °C for 10 min, followed by 32 cycles of denaturation at 94 °C for 30 s, annealing for 30 s and extension at 72 °C for 30 s. Cycling was followed by a final 7-min extension at 72 °C.

### Single-stranded conformation polymorphism (SSCP) analysis and sequencing mutational analysis

The PCR fragments were further analyzed by SSCP analysis using the DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, Inc.). The gel matrix for SSCP contained 8, 10 or 12% polyacrylamide gel (37.5:1; Bio-Rad Laboratories, Inc.) with or without 5% glycerol. Samples were electrophoresed for up to 20 h at a constant temperature of 5 °C. The gel was stained using the GelCode Color Silver Stain Kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Fragments with aberrant electrophoretic mobility were subsequently sequenced using the Big Dye terminator cycle sequencing kit and ABI PRISM 310 automated sequencer (PE Applied Biosystems). Exons 3, 7, 8, 9, 11 and 12 were directly sequenced in all

**Table 1** Clinical characteristics of mutation carriers and identified *TPO* gene mutations.

Family/ patient	Year of birth	Origin	TSH (mU/l) <sup>a</sup>	FreeT <sub>4</sub> (pmol/l) <sup>a</sup>	Total T <sub>4</sub> (nmol/l) <sup>a</sup>	Tg (ng/ml) <sup>a</sup>	TSH (mU/l) at reevaluation	Tg (ng/ml) at reevaluation	Thyroid gland <sup>b</sup>	<i>TPO</i> gene analysis results <sup>c</sup>
1	1999	Bosnia	> 80	ND	2.6	ND	NA	NA	Goiter	<b>613C&gt;T</b> /1273_1276dupGGCC
2	2003	Slovenia	186	2.4	ND	> 500	70.63	7.59	Congenital goiter <sup>d</sup>	1273_1276dupGGCC/WT
3/a	1983	Slovenia	> 80	ND	Not detectable	ND	NA	ND	Goiter	1273_1276dupGGCC/WT
3/b	1985	Slovenia	> 120	ND	16	ND	472	ND	Goiter	1273_1276dupGGCC/WT
4	1995	Slovenia	361	2.7	ND	209	40.20	102	Goiter	1273_1276dupGGCC/WT
5	2004	Slovenia	255.5	4.14	ND	180	53.59	75	Normal	1273_1276dupGGCC/WT
6	1996	Slovenia	13.1	16.2	ND	ND	16.7	10	Normal	1273_1276dupGGCC/WT
7	1990	Slovakia	64.4	ND	19.7	ND	NA	NA	Nodular goiter, follicular adenoma <sup>e</sup>	1273_1276dupGGCC/WT
8	1999	Bosnia	35.3	ND	0.008	88	NA	NA	Goiter	1273_1276dupGGCC/WT
9	1998	Slovenia	400	0.54	ND	2036	31.1	540	Goiter	1273_1276dupGGCC/ 1273_1276dupGGCC
10	1995	Slovenia	422	2.4	ND	664	122.60	530	Goiter	1273_1276dupGGCC/ 1273_1276dupGGCC
11	1996	Slovenia	603	13.9	ND	ND	78.90	231	Goiter	1273_1276dupGGCC/ 1273_1276dupGGCC
12/a	1986	Bosnia	> 60	ND	3.2	348	NA	NA	Goiter	1273_1276dupGGCC/ 2512delT
12/b	1998	Bosnia	> 60	ND	3.2	98	NA	NA	Goiter	1273_1276dupGGCC/ 2512delT
13	1991	Slovenia	> 500	ND	33	620	62.76	276	Goiter	<b>1519_1539del</b> /WT
14	1986	Slovenia	481	ND	10	ND	95.88	ND	Goiter	<b>1519_1539del</b> /WT
15	2003	Bosnia	306	ND	2.6	ND	NA	NA	Goiter	<b>1519_1539del</b> /WT
16	2003	Bosnia	99	ND	3.8	626	NA	NA	Goiter	<b>1519_1539del</b> /WT
17	1987	Slovenia	14.2	ND	155	368	7.40	ND	Nodular goiter <sup>f</sup>	<b>2089G&gt;A</b> /WT
18	1990	Slovenia	433.1	ND	16	ND	24.88	ND	Nodular goiter <sup>g</sup>	2485G>A/ <b>2669G&gt;A</b>
		Normal values	<8	11.7–28.0	59–163	2–70	0.35–5.50	2–70		

ND, not determined; NA, not available; WT, wild type.

<sup>a</sup>The serum was obtained on days 7–14 of life except in patient 3/a, where it was obtained at the age of 2.5 months.

<sup>b</sup>Ultrasound was performed at the age of 2 years unless indicated otherwise.

<sup>c</sup>Mutations in bold are novel.

<sup>d</sup>Ultrasound was performed in neonatal period.

<sup>e</sup>Ultrasound was performed at the age of 15 years.

<sup>f</sup>Ultrasound was performed at the age of 18 years.

<sup>g</sup>Ultrasound was performed at the age of 16 years.

patients, except in the three patients (nos 9, 10 and 11 in Table 1) where homozygosity for the common 1273\_1276dupGGCC mutation (A397PfsX76) was discovered using NaeI RFLP analysis. In 16 patients, all exons were sequenced. Sequences were compared with the normal *TPO* gene sequence (GenBank access no. AH003466).

### Genetic analysis of the *TPO* promoter

In participants, where only a single allele mutation was identified in the reading frame after the sequencing of all amplified exons, 873 bp of the promoter region were analyzed (GenBank access no. M25701:1727–2599) to examine for the presence of regulatory mutations. The region examined included the TATA box, CpG island, and the proximal promoter TTF-1- and PAX-8-binding sites, according to previous *TPO* promoter descriptions (34, 35). Three pairs of primers were designed for promoter region analysis, as listed: TP\_P1F, 5'-ttctgaagccttgcatcg-3'; TP\_P1R, 5'-cagagatgttctgtaggaaggtca-3'; TP\_P2F, 5'-tgaaagcagatcacccatga-3'; TP\_P2R, 5'-cctctggccgaatgagtag-3'; TP\_P3F, 5'-agttggtttattcttctcctgt-3'; and TP\_P3R, 5'-cggctgtaactcttctgaaatg-3'. Specific amplification conditions are available upon request at the corresponding author. The amplicons obtained by PCR were directly sequenced and the sequences were compared with the normal *TPO* promoter (GenBank access no. M25701).

### RFLP analysis

Screening for the common 1273\_1276dupGGCC mutation (A397PfsX76) was performed using NaeI RFLP analysis on a 2% agarose gel (11).

One hundred healthy controls (approximately 80% of Slovene ethnic origin and 20% of Bosnian ethnic origin) and examined healthy family members were screened for the novel mutations by RFLP on a 2% agarose gel. The novel point mutation in exon 6 (613C>T (R175X)) and a 1519\_1539del mutation (A477\_N483del) in exon 9 each eliminated one restriction site for endonuclease BsaXI. A silent mutation in exon 8 (930G>A (P280P or p.=)) eliminated the restriction site for endonuclease NaeI and the mutation in exon 11 (2089G>A (G667S)) eliminated the restriction site for HphI. However, a single nucleotide polymorphism placed immediately next to the mutation in exon 11 disturbed proper HphI digestions, therefore DNA samples with incomplete digestion were subsequently sequenced. A point mutation in exon 15 (2669G>A (G860R)) created a novel restriction site for BclI. Restriction enzymes used are summarized in Table 2. All restriction enzymes used were purchased from New England Biolabs, Inc., Beverly, MA, USA.

### Specific data analysis

For the novel mutations, phylogenetic conservation of the affected amino acid sequences among the human and mammalian *TPO* homologues was determined with the help of CLUSTAL program (<http://www.ebi.ac.uk/clustalw/>). For the silent alteration in exon 8, 930G>A (P280P or p.=), the possible effect on exonic splicing enhancers was assessed using the ESEfinder program (<http://rulai.cshl.edu/tools/ESE/>).

## Results

*TPO* gene mutations were detected in 20 out of 43 patients (46%) with thyroid dysmorphogenesis from 18 out of 39 families (46%). A similar prevalence was observed in Slovenians and Bosnians. Considering the incidence of 1:3100 of permanent CH in Slovenia, the percentage of dysmorphogenesis of 34%, and the prevalence of *TPO* mutations of 46% from the present study, the calculated incidence of CH due to *TPO* gene mutations in the Slovene population is approximately 1:20 000 newborns.

The *TPO* gene analysis results and patients' clinical characteristics are presented in Table 1. Mutations were numbered according to Kimura (10) and named as proposed by den Dunnen and Antonarakis (36). Two of the mutation carriers (nos 6 and 17 in Table 1) had mild hypothyroidism, while in the remainder initial laboratory values and the subsequent clinical course were indicative of a severe defect in thyroid hormone synthesis. Three patients (nos 7, 17 and 18) developed nodular goiter, with patient no. 7 undergoing right lobe surgical resection at the age of 15 years. Histological examination revealed nodular hyperplasia and follicular adenoma.

Seven different mutations were identified in the reading frame of the *TPO* gene, four of these being novel (613C>T in exon 6 (R175X), 1519\_1539del in exon 9 (A477\_N483del), 2089G>A in exon 11 (G667S), and 2669G>A in exon 15 (G860R); Fig. 1). Of the previously known mutations, 1273\_1276dupGGCC in exon 8 (A397PfsX76; (7, 11)), 2485G>A in exon 14 (E799K; (13)), and 2512delT in exon 14 (C808AfsX24; (7)) were detected. The most common mutation found in the examinees was the frequently reported inactivating mutation 1273\_1276dupGGCC (A397PfsX76), which was found in patients from all three countries, in 70% of mutation carriers and in 32% of all 43 participants. The identified mutations are summarized in Table 2.

In addition, two novel silent substitutions were identified. A substitution 930G>A in exon 8 (P280P or p.=) was detected in a heterozygous state in a patient with no other mutations detected. A substitution 2565C>T in exon 14 (C825C or p.=) was detected in a heterozygous state in one out of the two siblings,

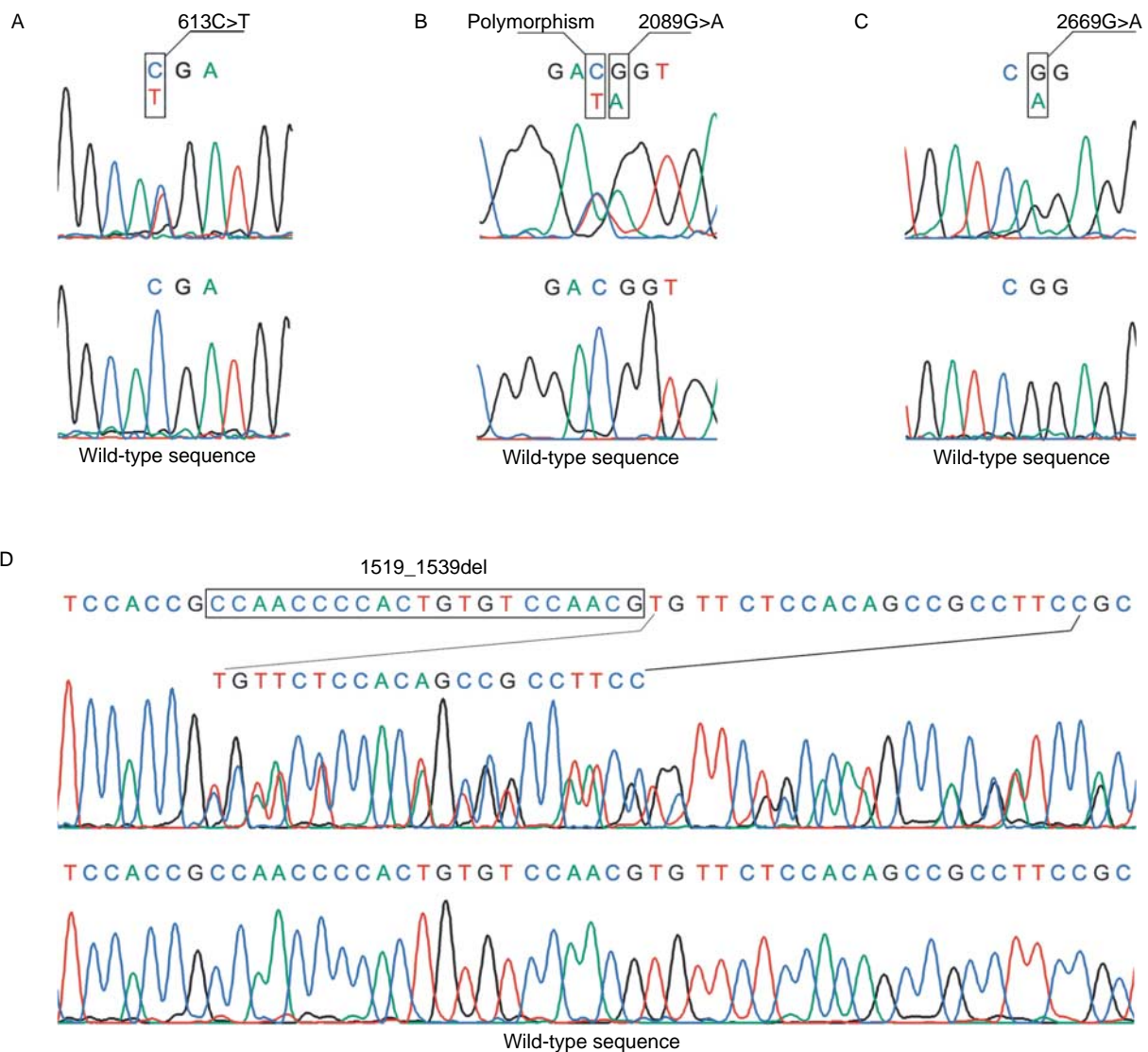
**Table 2** Mutations identified at the DNA and protein level, and restriction endonucleases used for RFLP.

Exon	Mutation <sup>a</sup>	Effect on TPO protein sequence	Reference	Restriction endonuclease	Frequency of mutant alleles
6	613C>T	R175X	Present study	BsaXI <sup>b</sup>	1/86
8	1273_1276dupGGCC	A397PfsX76	(11)	NaeI <sup>c</sup>	17/86
9	1519_1539del	A477_N483del	Present study	BsaXI <sup>c</sup>	4/86
11	2089G>A	G667S	Present study	HphI <sup>b</sup>	1/86
14	2485G>A	E799K	(14)		1/86
14	2512delT	C808AfsX24	(12)		2/86
15	2669G>A	G860R	Present study	BclI <sup>b</sup>	1/86

<sup>a</sup>Nucleotide numbering according to Kimura (10).

<sup>b</sup>Mutation eliminates restriction site.

<sup>c</sup>Mutation creates restriction site.



**Figure 1** Novel *TPO* gene mutations. (A) Heterozygous 613C>T mutation (R175X), wild-type exon 6 sequence. (B) Heterozygous 2089G>A mutation (G667S) and heterozygous 2088C>T single nucleotide polymorphism, wild-type exon 11 sequence. (C) Heterozygous 2669G>A mutation (G860R), wild-type exon 15 sequence. (D) Heterozygous 1519\_1539del mutation (A477\_N483del), wild-type exon 9 sequence.

both of whom had clinical signs of dysmorphogenesis. No mutations affecting the amino acid sequence were identified in any of the patients with silent mutations and these two patients were not included in the *TPO* gene mutation carriers. The 613T, 1519\_1539del, 2089A, 2669A, and 930A variants were not detected in any of the 100 healthy controls, either by RFLP analysis using specific restriction enzymes or by sequencing, which was used for the 2089A detection since RFLP analysis was not accurate due to the polymorphism placed next to the mutation.

Taking into consideration all the detected mutations, except for the two silent substitutions, seven patients were found to be homozygous or compound heterozygous *TPO* gene mutation carriers, while in 13 patients (65% of mutation carriers) only a single allele mutation was identified. In these patients, including the patient with the 930G>A silent substitution (P280P or p.=), all the exons analyzed by SSCP analysis were reexamined by sequencing and the *TPO* promoter region was investigated, except in patient no. 16 where additional DNA was not available for further analysis. In patient no. 17 a novel heterozygous promoter alteration –540g>a was identified; however, the analysis of family members showed that both this and the mutation 2089G>A in exon 11 (G667S) were inherited from the same parent.

Five known single nucleotide polymorphisms were detected in the promoter region and in exon 1 (–799g>a, –706g>a, –95g>t, –35a>g, and 11G>A) and 9 known polymorphisms in the reading frame (102C>G, L4L; 298C>G, P70A; 859G>T, A257S; 1207G>T, A373S; 1283G>C, S398T; 2088C>T, D666D; 2235C>T, P715P; 2263A>C, T725P; and 2630T>C, V847A). Among numerous identified intronic polymorphisms, the 96-52t>g single nucleotide polymorphism in intron 2 was detected in the heterozygous state in all four heterozygous carriers of a novel 1519\_1539del mutation in exon 9 (A477\_N483del; nos 13, 14, 15 and 16), but was not detected in any of the other study participants. In the parents of patient no. 13 the 96-52t>g polymorphism was shown to be inherited from the same parent as the 1519\_1539del mutation (A477\_N483del).

## Discussion

The prevalence of *TPO* gene mutations in the studied population (46%) was higher than that noted in a Portuguese study, where similar inclusion criteria, without the determination of the iodide organification defect, were used and mutations identified in 24% of patients (24). While the percentage of dysmorphogenesis (34%) in the Slovene population is higher than the 15% reported in other studies (2, 7), the estimated incidence of CH due to *TPO* gene mutations of 1:20 000 newborns is also considerably higher when compared with the published estimate in the Dutch population, where the calculated incidence of TIOD is 1:66 000 newborns (7). The data could be compared as the *TPO* gene mutations mainly result in TIOD, and most of the TIOD seem to be caused by *TPO* gene mutations (7).

In the present study, seven different *TPO* gene mutations were identified, four of these being novel. Mutations were also detected in two of the six participants with milder CH. In both patients (nos 6 and 17), only a single allele mutation was identified. In patient no. 6 the common 1273\_1276dupGGCC inactivating mutation (A397PfsX76) was detected. In patient no. 17 a novel missense mutation 2089G>A was found in exon 11 that caused an amino acid change from glycine with a nonpolar, hydrophobic residue, to serine, which is a polar and hydrophilic amino acid (G667S). Glycine in this position is highly conserved in human and mammalian *TPO* homologues (Table 3), Umeki *et al.* (23) published a functional study of the nearby mutation R665W, which was detected in a patient with severe CH. The mutation is shown to disturb plasma membrane localization in the mutated *TPO* protein. Considering the crystal structure of myeloperoxidase, the glycine corresponding to glycine 667 in *TPO* forms part of an  $\alpha$ -helix that interconnects two locally folded regions (37). Arginine 665 and glycine 667 form the same helical turn. Therefore, it is possible that this novel mutation reduces *TPO* enzyme function. However, in a recent publication, carriers of a single affected allele were not found to have any signs of thyroid dysfunction and they had normal iodide organification *in vivo* (29). Consequently, it is likely

**Table 3** Comparison of amino acid sequences neighboring novel mutations A447\_N483del and G667S in various human and mammalian peroxidases.

Mutant TPO	D	S	T	A <sup>477</sup>	N	P	T	V	S	N <sup>483</sup>	V	F	S	L	R	D	S <sup>667</sup>	D	W	F
Human TPO	D	S	T	A <sup>477</sup>	N	P	T	V	S	N <sup>483</sup>	V	F	S	L	R	D	G <sup>667</sup>	D	W	F
Pig TPO	D	P	A	V <sup>476</sup>	D	P	T	V	S	N <sup>482</sup>	V	F	S	L	R	D	G <sup>665</sup>	D	R	F
Mouse TPO	N	P	T	V <sup>465</sup>	N	P	T	V	S	N <sup>471</sup>	I	F	S	L	R	D	G <sup>655</sup>	D	R	F
Rat TPO	N	P	T	V <sup>465</sup>	N	P	T	V	S	N <sup>471</sup>	V	F	S	L	R	D	G <sup>655</sup>	D	R	F
Human MPO	N	D	S	V <sup>486</sup>	D	P	R	I	A	N <sup>492</sup>	V	F	–	L	R	D	G <sup>675</sup>	D	R	F
Mouse MPO	N	D	S	V <sup>460</sup>	D	P	R	I	A	N <sup>466</sup>	V	F	–	L	R	D	G <sup>649</sup>	D	R	F
Human EPO	C	S	N	V <sup>458</sup>	D	P	R	V	A	N <sup>465</sup>	V	F	–	A	R	D	G <sup>647</sup>	D	R	F
Mouse EPO	C	S	N	V <sup>459</sup>	D	P	R	V	A	N <sup>465</sup>	V	F	–	A	R	D	G <sup>648</sup>	D	R	F
Human LPO	S	E	S	V <sup>453</sup>	D	P	R	I	S	N <sup>459</sup>	V	F	–	L	R	D	G <sup>642</sup>	D	R	F
Mouse LPO	N	E	S	V <sup>451</sup>	D	P	R	I	S	N <sup>457</sup>	V	F	–	L	R	D	G <sup>640</sup>	D	R	F

TPO, thyroid peroxidase; MPO, myeloperoxidase; EPO, eosinophil peroxidase; LPO, lactoperoxidase.

that undetected intron or regulatory mutations affected the other allele in these two patients.

The functional importance of the three novel mutations that were found in patients with severe hypothyroidism could be predicted from the putative TPO protein structure. The 613C>T mutation creates a termination signal in exon 6 (R175X), causing a loss of the catalytic part of the enzyme.

A novel 21 bp deletion in exon 9, 1519\_1539del, causes a deletion of seven amino acids without a frame shift (A477\_N483del). This short amino acid sequence is partly conserved among other mammalian peroxidases, as shown in Table 3. Several other mutations causing TIOD have been identified in close proximity by other groups – I447F, Y453D, L458P, R491H, G493S, and P499L (13, 19, 20, 22, 25). Exons 8, 9 and 10 encode for the catalytic part of the enzyme and are the most frequent locations of inactivating mutations. Histidine 494 in human TPO is, according to the known myeloperoxidase structure, the proximal heme-binding histidine (37). A nearby deletion of seven amino acids would be expected to disturb proper protein folding and the final structure. The 1519\_1539del mutation was detected in four apparently unrelated patients. Two of these were Bosnians, nos 15 and 16, and the other two had Slovene citizenship, nos 13 and 14, but their families had previously migrated to Slovenia from the southern republics of the former Yugoslavia. The nucleotide sequence of a deleted oligonucleotide and the nucleotides that directly surround it did not show the properties of loop formation as a possible mechanism for a deletion that would arise *de novo* on multiple occasions. However, the linkage of the deletion with an intronic substitution 96-52t>g, detected only in these four patients, implies the same ancestral origin of the mutation.

The 2669G>A (G860R) is, to our knowledge, the first mutation resulting in an amino acid substitution in the TPO transmembrane region (38). It is very likely that the change from the hydrophobic glycine to the positively charged arginine disturbs the insertion of the TPO enzyme into the plasma membrane.

The functional importance of the previously known mutations was investigated by other groups. The 1273\_1276dupGGCC mutation is a frame shift mutation that creates a premature termination signal in exon 9 (A397PfsX76), but is also shown to unmask a cryptic acceptor splice site in exon 9. Through the mechanism of alternative splicing normal reading frame is restored in the carboxy-terminal half of the protein (11). The 2485G>A mutation (E799K) results in enzymatically inactive TPO, while the cellular distribution of this mutant is normal (20). The 2515delT frame shift mutation in exon 14 generates a premature termination signal in the same exon (C808AfsX24; (7)). Expression analysis showed that the mutated TPO is smaller and cannot translocate to the cell surface (39).

A novel heterozygous substitution 930G>A (P280P or p.=), situated 20 bp inside exon 8 seems to be functionally silent. It lies 20 bp inside the exon. The computer program ESEfinder's calculations showed that this alteration eliminated two high-score exonic splicing enhancer sites, Sf2/Asf (score 4.175) and Sc35 (score 3.234). This mutation was not detected in 100 healthy controls. However, the possible effect on exon splicing could not be verified, because no thyroid tissue from this patient was available for mRNA analysis. The other novel silent substitution, 2565C>T (C825C or p.=), was shown not to segregate with the disease.

In an unexpectedly high percentage of patients (65%), only single allele mutations were identified, despite the fact that all 17 exons were analyzed in these patients, the normal SSCP analysis results were confirmed by subsequent sequencing and, in addition, 873 bp of the proximal promoter were analyzed. In other studies, single TPO mutations were detected in only about 20% of patients with TIOD (7, 22, 40, 41). One report of mRNA analysis after thyroidectomy in a patient with TIOD and a single allele mutation proved a lack of expression of the other allele with a normal reading frame. No proximal promoter mutation was found in this patient (40).

TPO gene mutations are mostly inactivating (7). However, there are reports about TPO gene mutations that only partly diminish TPO function and result in milder clinical presentation (30). No phenotype-genotype correlation was appreciated in patients with mutations identified on both alleles. Two patients with a single allele mutation had milder clinical presentation of CH, however, additional mutations in intronic regions or unexamined parts of regulatory regions were possible.

In conclusion, the prevalence of TPO gene mutations was considerably higher when compared with other published data. Four novel TPO gene mutations were identified in the examined population. Considering the high prevalence of single allele TPO mutations, the overall prevalence of TPO mutations with possible unidentified cryptic mutations could be even higher.

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