Mild thyroid peroxidase deficiency caused by *TPO* mutations with residual activity: Correlation between clinical phenotypes and enzymatic activity

Satoshi Narumi¹⁾, Larry A Fox²⁾, Keisuke Fukudome^{3),4)}, Zenichi Sakaguchi³⁾, Chiho Sugisawa⁵⁾, Kiyomi Abe⁵⁾, Kaori Kameyama⁶⁾ and Tomonobu Hasegawa⁵⁾

¹⁾ Department of Molecular Endocrinology, National Research Institute for Child Health and Development, Tokyo 157-8535, Japan

²⁾ Division of Endocrinology, Nemours Children's Specialty Care, Jacksonville, FL 32207, USA

³⁾ Department of Pediatrics, Takamatsu Red Cross Hospital, Takamatsu 760-0017, Japan

⁴⁾ Department of Pediatric Cardiology, Shikoku Medical Center for Children and Adults, Zentsuji 765-8507, Japan

⁵⁾ Department of Pediatrics, Keio University School of Medicine, Tokyo 160-8582, Japan

⁶⁾ Department of Diagnostic Pathology, Keio University School of Medicine, Tokyo 160-8582, Japan

Abstract. Thyroid peroxidase (TPO) deficiency, caused by biallelic *TPO* mutations, is a well-established genetic form of congenital hypothyroidism (CH). More than 100 patients have been published, and the patients have been diagnosed mostly in the frame of newborn screening (NBS) programs. Correlation between clinical phenotypes and TPO activity remains unclear. Here, we report clinical and molecular findings of two unrelated *TPO* mutation-carrying mildly hypothyroid patients. The two patients were born at term after an uneventful pregnancy and delivery, and were NBS negative. They sought medical attention due to goiter at age 8 years. Evaluation of the thyroid showed mild elevation of serum TSH levels, normal or slightly low serum T4 levels, high serum T3 to T4 molar ratio, high serum thyroglobulin levels, and high thyroidal ¹²³I uptake. We performed next-generation sequencing-based genetic screening, and found that one patient was compound heterozygous for two novel *TPO* mutations (p.Asp224del; c.820-2A>G), and the other was homozygous for a previously known mutation (p.Trp527Cys). *In vitro* functional analyses using HEK293 cells showed that the two amino acid-altering mutations (p.Asp224del and p.Trp527Cys) caused partial loss of the enzymatic activity. In conclusion, we report that *TPO* mutations with residual activity are associated with mild TPO deficiency, which is clinically characterized by marked goiter, mild TSH elevation, high serum T3 to T4 molar ratio, and high serum thyroglobulin levels. Our findings illuminate the hitherto under-recognized correlation between clinical phenotypes and residual enzymatic activity among patients with TPO deficiency.

Key words: Congenital hypothyroidism, Newborn screening, Thyroid peroxidase (TPO), Mutation, Genetics

CONGENITAL HYPOTHYROIDISM (CH) is the most common congenital endocrine disorder affecting about 1 in 2,000-4,000 newborns [1]. CH is recognized as one of the most common causes of preventable intellectual disability. In virtually all developed countries, newborn screening (NBS) programs were implemented to enable early diagnosis and treatment, hereby preventing intellectual disability. As a result, the vast

majority of CH patients, including mild ones, are diagnosed in the frame of NBS in those countries.

Thyroid peroxidase (TPO) is a thyroid-specific membrane-bound enzyme that plays a crucial role in production of the thyroid hormones. TPO oxidizes iodide ions to form iodine atoms for addition onto tyrosine residues on thyroglobulin (Tg) for the production of iodotyrosine residues (mono-iodinated tyrosine or di-iodinated tyrosine). TPO also catalyzes the coupling reaction of two iodotyrosine residues, ultimately forming T₃ or T₄ residues in Tg. TPO deficiency is a well-established genetic form of CH. In 1950, the disease was originally described by Stanbury and Hedge [2] as an autosomal-recessive form of CH characterized by inability to utilize iodine in the thyroid.

Submitted May 4, 2017; Accepted Jul. 24, 2017 as EJ17-0194

Released online in J-STAGE as advance publication Sep. 1, 2017

Correspondence to: Satoshi Narumi, M.D., Ph.D., Department of Molecular Endocrinology, National Research Institute for Child Health and Development, 2-10-1 Okura, Setagaya-ku, Tokyo 157-8535, Japan.

E-mail: narumi-s@ncchd.go.jp

Identification of the disease-causing mutations involving the TPO gene (*TPO*) was achieved by Abramowicz *et al.* in 1992 [3]. Since then, more than 100 molecularly-confirmed cases harboring more than 70 distinct mutations have been published worldwide [4]. Likely due to the presence of a common founder frameshift mutation, referred as “GGCC insertion in exon 8” in the literature [3], the TPO deficiency seems more frequently observed in Caucasians than in other ethnic groups. A genetic epidemiology study conducted in the Netherlands showed that the frequency of the deficiency was 1 in 66,000 among the general Dutch populations, accounting for roughly 3% of total CH cases [5]. The classical hallmark of the TPO deficiency is total iodine organification defect, in which more than 90% of accumulated intrathyroidal radioiodine is released after administration of perchlorate [5]. A minor subset of *TPO* mutation carriers have partial iodine organification defect, though the frequency of the phenotype remains unknown.

Whether iodine organification is total or partial, TPO deficiency is congenital, and the disease is lifelong (*i.e.*, permanent CH). Thus, it seems natural that hypothyroidism in *TPO* mutation carriers can readily be detected through the NBS programs. Here, we report two *TPO* mutation-carrying mildly hypothyroid patients that

had negative NBS results. The two unrelated patients came to medical attention due to goiter, had a mildly elevated serum TSH level, and exhibited characteristic high serum T₃ to T₄ molar ratio. Functional analyses revealed that the mutant TPO proteins retained significant residual peroxidase activity, likely explaining the milder phenotypes than most previous cases.

Materials and Methods

Patients

Patient 1, the third child of healthy non-consanguineous Japanese parents, was a female born at term after an uneventful pregnancy and delivery. She received TSH-based NBS. The result, reported by the parents, was negative. She developed goiter at age 8 years. She had normal height (126.0 cm; -0.2 SD) and normal BMI (z-score, -0.6). No symptoms or signs suggesting hypothyroidism were noted. She had markedly large thyroid gland, which was soft to palpation. Blood test showed a slightly elevated serum TSH (6.5 mU/L, reference 0.5–5.0), normal thyroid hormones (free T₄ 0.99 ng/dL, reference 0.90–1.80; free T₃ 4.1 pg/mL, reference 2.6–4.9) with an elevated serum Tg (1,057 ng/mL; reference <30 ng/mL) (Fig. 1). Serum levels of anti-TPO antibody and anti-Tg antibody were normal. On

Fig. 1 Chronological changes of serum TSH level, serum free T₄ levels, and serum T₃ to T₄ molar ratio (free T₃/free T₄ in Patient 1, total T₃/total T₄ in Patient 2) are shown. Shaded areas indicate reference ranges. The two patients had mildly elevated TSH levels and characteristic high T₃/T₄ molar ratio.

Abbreviations: FT₃, free T₃; FT₄, free T₄; LT₄, levothyroxine replacement therapy; TT₃, total T₃; TT₄, total T₄.

ultrasonography, the thyroid gland was not only very large (estimated thyroid volume, 68 mL) but also showed increased vascularity (Fig. 2A). She had high thyroidal ^{123}I uptake (65% at 4 h; reference 5–15%), and the perchlorate discharge rate was high (35.4%; reference <10%). She was treated with levothyroxine. FT_3 to FT_4 molar ratio was high at the diagnosis of subclinical hypothyroidism (0.49, in-house reference 0.33–0.41), normalized after starting therapy, but became constantly high after age 12 years (Fig. 1). Since the initiation of treatment, progression of goiter was stopped. There were no recognizable abnormalities in growth, intelligence and pubertal development.

Patient 2, an 8-year-old Caucasian boy born in USA, sought medical attention for goiter. He had mild

speech delay but growth in height was normal. The result of NBS, reported by the parents, was negative. His family history was positive for maternal grandfather and maternal great uncle both having thyroid cancer (tissue types unknown). On physical examination, his enlarged thyroid gland was soft and asymmetric ($\text{R}>\text{L}$). Thyroid function test showed a slightly high serum TSH (5.9 mU/L) (Fig. 1), a low total T_4 (3.5 $\mu\text{g}/\text{dL}$; reference 6.0–13.8), and a high total T_3 (2.20 ng/mL; reference 0.60–1.81). He had slightly high serum Tg (250 ng/mL). Serum levels of anti-TPO antibody and anti-Tg antibody titers were normal. ^{123}I uptake was elevated (72% at 4 h). Perchlorate discharge test was not performed. His goiter was not improved by levothyroxine treatment, and actually worsened. He

Fig. 2 Thyroid morphology and histology of Patient 1 and Patient 2

(A) Ultrasonography showed marked goiter in Patient 1 (left panel), with increased vascularity on color Doppler ultrasound (right panel). (B) Photographic image of resected thyroid gland of Patient 2. (C) Histological analysis of the resected thyroid specimen showed irregular and distended macrofollicles. (D) Immunohistochemical staining of TPO showed no significant difference between Patient 2 (left panel) and a control (right panel).

received thyroidectomy at age 17 years (the weight of the resected gland was 102 g, Fig. 2B). Histological analysis showed irregular and distended macrofollicles with scattered delicate papillary infoldings, dense colloid, and absence of resorption vacuoles (Fig. 2C). Immunohistochemical stain showed expression of the TPO protein in the thyroid follicular cells that was comparable to a control (Fig. 2D). Simultaneous measurements of T₃ and T₄ were conducted three times, when the patient was not treated, and very high serum total T₃ to total T₄ molar ratio (0.075, 0.064 and 0.080; reference 0.010–0.027)[6] was observed.

Mutation detection

This study was approved by the Ethics Committee of Keio University School of Medicine. Written informed consent for the molecular study was obtained from the parents or legal guardians of the probands. Genomic DNA samples were collected from the probands and family members if available. Ten known genes associated with CH (*DUOX2*, *DUOX2*, *IYD*, *NKX2-1*, *PAX8*, *SLC5A5*, *SLC26A4*, *TG*, *TPO* and *TSHR*) were analyzed with use of a next generation sequencer MiSeq (Illumina Inc., San Diego, CA, USA) according to the SureSelect protocol (Agilent Technologies, Santa Clara, CA, USA) as described previously [7]. Base calling, read filtering, and demultiplexing were performed with the standard Illumina processing pipeline. We used BWA 0.7.5 for alignment against the human reference genome (NCBI build 37; hg19) with the default settings. Local realignment, quality score recalibration and variant calling were performed by GATK 3.2.0 [8] with the default settings. We used ANNOVAR [9] for annotation of called variants. The detected mutation was confirmed by standard PCR-based Sanger sequencing with previously described methods [10]. We referred the frequencies of the mutations in the variant databases, including 1,000 Genomes Project (1KG; <http://www.internationalgenome.org/>), Exome Aggregation Consortium (ExAC; <http://exac.broadinstitute.org/>) and Human Genetic Variation Database (HGVD; <http://www.genome.med.kyoto-u.ac.jp/SnpDB/>). Descriptions of the mutations were based on NM_000547.5.

Functional analyses

A vector encoding C-terminal hemagglutinin-tagged human TPO (TPO-HA) was created by insertion of TPO cDNA sequence into pEGFP-N1 (Clontech lab-

oratories, Palo Alto, CA), and substitution of the EGFP sequence by the HA sequence with the Gibson assembly technique (NEBuilder HiFi DNA Assembly Master Mix; New England Biolabs, Ipswich, MA, USA). The two TPO mutations (p.Asp224del, and p.Trp527Cys) were introduced by site-directed mutagenesis (TaKaRa Bio Inc, Tokyo, Japan). Expression vectors for stable expression of (untagged) TPO [wildtype (WT), p.Asp224del, p.Trp527Cys and p.Arg341Gln] were created by insertion of TPO cDNA sequence into pB513B-1 (System Biosciences, Palo Alto, CA, USA) with the Gibson assembly technique.

HEK293 cells were maintained in DMEM supplemented with 50 U/mL penicillin, 50 µg/mL streptomycin and 10% fetal bovine serum. In a series of expression experiments, we assessed a previously reported TPO mutants (p.Arg341Gln [10]) as a representative of nonfunctional mutation. We also analyzed the p.Gly387Arg mutation that was observed in patients showing the mild TPO deficiency phenotype [11]. Stable HEK293 cell lines expressing each TPO protein (WT, p.Asp224del, p.Trp527Cys, p.Gly387Arg and p.Arg341Gln) were established using the piggyback system according to the manufacturer's protocol. For immunoblot analysis, stable cells were grown in a 6-well plate to about 95% confluency, washed twice with PBS, and lysed with 200 µL of 0.5% Triton-X100 in PBS. Cell lysates were subject to 10% SDS-PAGE, and Western blot was performed with use of the anti-TPO rabbit monoclonal antibody ab109383; Abcam, Cambridge, MA, USA) as a primary antibody. For measurement of peroxidase activity, we established stable cell lines expressing each TPO protein (WT, p.Asp224del, p.Trp527Cys, p.Arg341Gln and p.Gly387Arg) according to the manufacturer's protocol. We prepared 90% confluent stable cells in 6-cm dishes, trypsinized and washed cells twice with PBS, and resuspended them in 200 µL of 1× Earle's balanced salt solution (Sigma Aldrich, St. Louis, MO, USA) containing 100 µM Amplex Red reagent (Thermo Fisher Scientific) and 2 mM H₂O₂. After 1-hour shaking incubation at room temperature, fluorescent emission was measured with the FlexStation 3 Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). The assay was performed to quantify the peroxidase activity of the stable HEK293 cells. When we perform the assay for WT-TPO expressing cells with permeabilizing plasma membrane, increase of the activity (about 15%) was observed, indicating that

the peroxidase activity was chiefly derived from the cell surface. The activity of each mutant is expressed as a percentage (mean $\pm$ SEM) of WT activity. The background activity, which was measured with use of non-transfected cells, was set to 0%. Data are representative of three independent experiments (each performed in triplicate) with similar results. Welch's *t*-test was used for comparisons of the activity.

Results

Mutation detection

The next generation sequencing-based genetic screen led us to identify biallelic mutations in both patients. Patient 1 was compound heterozygous for a

novel 3-bp deletion causing a loss of one amino acid residue Asp224 (c.670_672del, p.Asp224del) (Fig. 3A). The Asp224 residue is conserved among mammalian species (Fig. 3B). She also had a novel splice site mutation, c.820-2A>G, affecting the boundary of intron 6 and exon 7 (Fig. 3A). The c.820-2A>G mutation was derived from the father, while the p.Asp224del mutation was derived from the mother (Fig. 3C). The elder sister of the proband had the p.Asp224del mutation in the heterozygous state. Patient 2 was homozygous for a previously reported missense mutation (Fig. 3A), p.Trp527Cys [5], affecting the evolutionarily conserved amino acid residue (Fig. 3B). Parental genotyping was unavailable.

The c.820-2A>G splice site mutation was not

Fig. 3 Identification of *TPO* mutations

(A) Partial electropherograms of the three identified *TPO* mutations. Patient 1 was compound heterozygous for a novel one amino-acid deletion (p.Asp224del, the deleted nucleotides are indicated in a box) and a novel splice site mutation (c.820-2A>G, red arrow). Patient 2 had a previously known missense mutation (p.Trp527Cys, red arrow) in a homozygous state. (B) The residues affected by the amino acid-altering mutations (Asp²²⁴ and Trp⁵²⁷) were conserved across the mammalian species (*i.e.*, human, mouse and rat). (C) Pedigrees of the Patient 1 (left) and Patient 2 (right). The two patients were sporadic. Values presented below each symbol indicate thyroid function test results. Squares, men; circles, women; solid symbols, affected by hypothyroidism; open symbols, unaffected. Abbreviations: FT₃, free T₃; FT₄, free T₄; NA, not available; Tg, thyroglobulin; WT, wildtype.

registered in 1KG, ExAC or HGVD. The p.Asp224del and the p.Trp527Cys mutations were registered in the ExAC database with extremely low allele frequency (0.000057 and 0.00010, respectively).

Functional analyses

To confirm the pathogenicity of previously uncharacterized amino acid-altering mutations (p.Asp224del and p.Trp527Cys), we conducted *in vitro* expression experiments. The protein expression levels of the mutants, which were evaluated by transient transfection and Western blotting, were comparable to that of WT protein (Fig. 4A). The *in vitro* observation of the p.Trp527Cys mutation was consistent with the *in vivo* observation in Patient 2, which showed normal TPO immunostaining (Fig. 2D). We generated HEK293 cell lines that stably express each TPO protein (WT or mutant), and cell surface peroxidase activity were measured with use of Amplex Red reaction. We found that the p.Asp224del mutant and the p.Trp527Cys mutant had partial peroxidase activity, which were $56 \pm 6\%$ ($p=0.012$) and $31 \pm 3\%$ ($p<0.001$) of WT, respectively (Fig. 4B). In this assay, a previously reported nonfunctional mutation p.Arg341Gln [10] showed negligible activity (Fig. 4B). We also analyzed a previously reported but uncharacterized mutation p.Gly387Arg, which had been observed

in CH patients with negative NBS results, and confirmed that the mutant also retained residual activity (Fig. 4B).

Discussion

In this report, we described two *TPO* mutation carriers that had negative NBS results, marked goiter, mildly elevated serum TSH levels high serum T₃ to T₄ molar ratio, and elevated serum Tg. Expression experiments revealed that the two amino-acid altering mutations (p.Asp224del and p.Trp527Cys) were loss-of-function mutations, but retained significant residual enzymatic activity. We presume that the two patients had mild TPO deficiency.

After the implementation of NBS programs, the vast majority of *TPO* mutation carriers have been diagnosed *via* NBS. To our knowledge, only five mutation-carrying patients (belonging to two families) that were missed by NBS have been reported. Kotani *et al.* described three siblings with compound heterozygous *TPO* mutations (p.[Gly553Cys];[Asp574_Leu575del]) [12]. Presenting symptom of the first and second children was goiter at ages 12 and 8 years, respectively. The third child had no symptoms, but received ultrasonography at age 8 months due to the family history, and goiter was shown. Lee *et al.* described three siblings with a homozygous *TPO* mutation (p.Gly387Arg)[11].

Fig. 4 Functional characterization of the identified amino acid-altering *TPO* mutations (p.Asp224del and p.Trp527Cys)

The p.Arg341Gln mutation was analyzed as a representative of nonfunctional mutations. The p.Gly387Arg mutation, which had been observed in patients with the mild TPO deficiency phenotype was also analyzed. (A) Western blotting showed no significant changes in protein expression levels between the mutants and wildtype. The image is a representative of three independent experiments with similar results. (B) Peroxidase activity of the p.Asp224del mutant and the p.Trp527Cys mutant were significantly lower than the wildtype ($56 \pm 6\%$ and $31 \pm 3\%$, respectively), though residual activity were observed. The p.Arg341Gln mutant was nonfunctional. The p.Gly387Arg mutation retained residual activity. Data are representative of three independent experiments, each performed in triplicate, with similar results. Bars indicate standard error of the mean.

Among the three affected siblings, the proband (fourth child) was diagnosed as having CH in the frame of NBS, while the remaining two (first and third children) had negative NBS results and developed goiter at ages 19 and 12 years, respectively. The five NBS-negative patients in the literature and our two patients had very mild elevation of serum TSH levels (3.8–9.2) (Table 1), and thus the levels did not seem to reach the screen cutoff. An alternative explanation for the negative NBS is delayed elevation of serum TSH in these patients. However, considering that the proband of the family with the homozygous p.Gly387Arg mutation had a positive NBS result, it is more likely that TSH was also elevated in the NBS-negative patients in their newborn periods, but the degrees were mild. However, this cannot be proven in the absence of thyroid function test data in the newborn period.

False negative NBS results are not specific to the mild TPO deficiency but also occur in patients with mild CH. The characteristic finding was high serum T₃ to T₄ molar ratio, observed not only in our two patients but also in three siblings reported by Kotani *et al.* (FT₃ to FT₄ molar ratio: 0.47, 0.47 and 0.67) (Table 1). The T₃ to T₄ molar ratio values in Patient 1 correlated well with serum TSH levels ($R^2=0.48$, $p=0.0028$) and high T₃ to T₄ molar ratio was observed when serum TSH levels were also high. This TSH-dependent elevation

of T₃ to T₄ molar ratio indicates that increased T₃ is derived from the thyroid (but not converted from T₄ in peripheral tissues), because secretion of T₃ from the thyroid varies depending on serum TSH levels [13]. Similar high T₃ to T₄ molar ratio phenotype has also been described in patients with adult-onset Tg deficiency [14–16]. In patients with Tg deficiency, increased thyroidal type 2 iodothyronine deiodinase activity was shown by measurements of the enzymatic activity of frozen thyroid resection samples. The upregulation of type 2 iodothyronine deiodinase suggests that the mutation-carrying thyroid glands secrete T₃ more preferentially than usual, possibly an adaptation to the Tg deficiency. We presume similar adaptive response might occur in patients with mild TPO deficiency, but a lack of frozen samples prevents us from performing verification studies.

Although more than 70 TPO mutations have been reported, *in vitro* determination of enzymatic activity has been performed only for 12 mutations (Supplementary Table 1)[10, 12, 17–19]. Ten out of the 12 mutations had no measurable activity, while two (p.Asp574_Leu575del and p.Gly590Ser) had residual activity [12, 17]. The p.Gly590Ser mutation had near-normal enzymatic activity, although it was observed in a patient with total iodine organification defect [17]. The discrepancy between experimental and clinical

Table 1 Phenotypes of mild TPO deficiency

Variables	Family A-1 [12]	Family A-2 [12]	Family A-3 [12]	Family B-1 [11]	Family B-2 [11]	Family C-1 ^a	Family D-1 ^a	Reference
<i>TPO</i> genotype	p.G553C p.D574_L575del	p.G553C p.D574_L575del	p.G553C p.D574_L575del	p.G387R (homozygous)	p.G387R (homozygous)	p.D224del c.820-2A>G	p.W527C (homozygous)	
Sex	M	M	F	F	F	F	M	
Age at diagnosis (year)	12	8	0 ^b	19	12	8	8	
Presenting symptom	Goiter	Goiter	Goiter ^b	Goiter	Goiter	Goiter	Goiter	
Serum TSH (mU/L)	4.0	6.0	9.2	5.4^c	3.8 ^c	6.5	5.9	0.5–5.0
Serum free T ₄ (ng/dL)	0.9	0.7	1.3	1.2 ^c	0.9 ^c	1.0	NA	0.9–1.8
Serum free T ₃ (pg/mL)	3.7	4.0	5.3	NA	NA	4.1	NA	2.6–4.9
Free T ₃ /free T ₄ molar ratio	0.47	0.67	0.47	NA	NA	0.48	NA	0.33–0.41
Serum Tg (ng/mL)	123	129	577	NA	999	1,057	250	<30
¹²³ I uptake (% _h)	35 , 3	35 , 3	NA	NA	NA	65 , 4	72 , 4	5–15 ^d
KClO ₄ discharge rate (%)	84	76	NA	NA	NA	35	NA	<10

^a Present report. ^b The patient had no symptoms, but received ultrasonography at age 8 months due to the familial history, and goiter was shown. ^c Data obtained through personal communication from Dr Muhammad Yazid Jalaludin (Department of Paediatrics, Faculty of Medicine, University of Malaya, Malaysia). ^d Reference range for ¹²³I uptake at 4 h.

data could be due to the location of the p.Gly590Ser mutation (the last nucleotide of exon 10; reference sequence: NM_000547), because the mutation could prevent normal splicing and result in truncation of the gene product. The p.Asp574_Leu575del mutation retains about 50% enzymatic activity as compared with WT, and was observed in three NBS-negative siblings (*i.e.*, mild TPO deficiency)[12]. In the present study, we showed that the two amino-acid altering mutations (p.Asp224del and p.Trp527Cys) retains significant residual activity, and were associated with a mild phenotype. The p.Asp224del mutation has about 50% activity as compared with WT. Considering that the other *TPO* allele was seemingly a nonfunctional splice site mutation (c.820-2A>G), the total TPO activity of Patient 1 is approximately 25%, on assumption that both alleles are expressed at the same level. The peroxidase activity of the other mutation, p.Trp527Cys, was about 30% as compared with WT. Patient 2 was homozygous for the p.Trp527Cys mutation, and thus the total TPO activity is expected to be approximately 30%. Of interest, heterozygous p.Trp527Cys mutation, along with heterozygous p.Gln446His mutation, has been observed in three siblings with severe permanent CH [20], indicating that the p.Trp527Cys mutation can produce both mild and severe phenotype, depending on the activity of the opposite allele.

The two patients described in the present report had very large goiter, although their serum TSH levels were only modestly elevated. Obviously, size of thyroid gland in untreated thyroid dysmorphogenesis varies depending on not only serum TSH levels but also duration of hyperthyrotropinemia. Thus, the long untreated duration (8 years) may explain the development of very large goiter. Moreover, we presume that direct effect(s) of the mutant TPO proteins contributed goiter development. The size of thyroid gland of the two patients was not completely reversed after initiation of levothyroxine treatment, implying direct and TSH-independent effect(s) of the mutant proteins. Through analyses of thyroid tissues derived from Tg-deficient patients, Baryshev *et al.* proposed that unfolded protein response triggered by mutant Tg proteins play a role in goiter development [21]. Similar mechanisms could be valid for TPO mutations.

Environmental factors, such as iodine intake level, are known to modify the clinical pictures of both genetic and non-genetic hypothyroidism. In some forms of thyroid dysmorphogenesis, including

sodium-iodine symporter defect [22] and dual oxidase 2 defect [23], excessive amount of iodine seems to rescue the thyroid hormone-producing capacity. The two *TPO* mutation-carrying patients described in the present study lived in iodine-sufficient countries (Japan and USA), possibly let the clinical phenotype relatively mild.

Collectively, we suggest a previously under-recognized correlation between residual enzymatic activity and clinical phenotypes. It has long been known that biallelic nonfunctional *TPO* mutations (total TPO activity ranging from 0 to about 15%) produce iodine organification defect, while monoallelic (*i.e.*, heterozygous) mutation carriers (total TPO activity, ~50%) have normal thyroid function (Supplementary Fig. 1). Here, we define a new subtype of TPO deficiency, mild TPO deficiency, caused by *TPO* mutations with residual activity (total TPO activity, about 30%). The typical phenotypes of mild TPO deficiency includes marked goiter, mildly elevated increased TSH levels, high serum T₃ to T₄ molar ratio, and elevated serum Tg levels.

Acknowledgments

The authors thank Ms. Saori Miyasako for the technical assistance; Drs Muhammad Yazid Jalaludin and Sarni binti Mat Junit (University of Malaya, Malaysia) for providing us the unpublished clinical information. This work was partly supported in part by JSPS KAKENHI Grant Number JP15K09630 from the Japan Society for the Promotion of Science, and a grant from the Ministry of Health, Labour and Welfare, Japan (Jitsuyoka [Nanbyo]-Ippan-014).

Disclosure

The authors have no financial relationships relevant to this article to disclose.

Author contributions

S.N. and T.H. designed the project. S.N., C.S. and K.A. conducted sequencing analyses. S.N. carried out *in vitro* experiments. L.A.F., K.F. and Z.F. and H.K. clinically characterized the patients and collected biological samples. K.K. conducted histological analysis. S.N. wrote the manuscript, and L.A.F. edited the manuscript.

Supplementary Table 1 *In vitro* functional activities of TPO mutants

TPO mutation	Functional analysis			Clinical information	
	Cells; transfection	Assay methods	Residual activity	Clinical phenotype	The opposite allele
Asp224del ^a	HEK293; stable	Amplex Red	Present	Nonautoimmune hypothyroidism	c.820-2A>G
Asp240Asn ^b	CHO-K1; transient	Guaiacol oxidation	Absent	CH (partial iodine organification defect)	p.Cys808fs
Arg341Gln ^c	HEK293; transient	Amplex Red	Absent	CH (organification unknown)	p.Gly860Arg
Gly387Arg ^d	HEK293; stable	Amplex Red	Present	Nonautoimmune hypothyroidism	Homozygous
Ile447Phe ^e	CHO-K1; transient	Guaiacol oxidation	Absent	CH (total iodine organification defect)	Unknown
Tyr453Asp ^e	CHO-K1; transient	Guaiacol oxidation	Absent	CH (total iodine organification defect)	Homozygous
Trp527Cys ^a	HEK293; stable	Amplex Red	Present	Nonautoimmune hypothyroidism	Homozygous
Gly553Cys ^b	CHO-K1; transient	IF and guaiacol oxidation	Absent	Nonautoimmune hypothyroidism	p.Asp574_Leu575del
Asp574_Leu575del ^b	CHO-K1; transient	IF and guaiacol oxidation	Present	Nonautoimmune hypothyroidism	p.Gly553Cys
Gly590Ser ^{e,f}	CHO-K1; transient	Guaiacol oxidation	Present	CH (total iodine organification defect)	Homozygous
Arg665Trp ^g	CHO-K1; transient	Guaiacol oxidation	Absent	CH (organification unknown)	p.Gly771Arg
Gly771Arg ^g	CHO-K1; transient	Guaiacol oxidation	Absent	CH (organification unknown)	p.Arg665Trp
Glu799Lys ^e	CHO-K1; transient	Guaiacol oxidation	Absent	CH (total iodine organification defect)	p.Glu17fs
Cys808fs ^e	CHO-K1; transient	Guaiacol oxidation	Absent	CH (total iodine organification defect)	Homozygous
Gly860Arg ^c	HEK293; transient	Amplex Red	Absent	CH (organification unknown)	p.Arg341Gln

Abbreviations: CH, congenital hypothyroidism; IF, immunofluorescence.

^a Present report. ^b Kotani T *et al.*, *J Endocrinol* 1999 160 267-273. ^c Narumi S *et al.*, *J Clin Endocrinol Metab* 2011 96 E1838-1842.^d Lee CC *et al.*, *Horm Res Paediatr* 2014 81 356-360. ^e Bikker H *et al.*, *J Clin Endocrinol Metab* 1997 82 649-653. ^f This mutation is located in the exonic splice site. ^g Umeki K *et al.*, *Eur J Endocrinol* 2002 146 491-498.

	Classification	Phenotypes	Representative genotype
100%	Mutation non-carriers	Normal TSH Normal thyroid size	WT (homozygous)
50%	Monoallelic mutation carriers	Normal TSH Normal thyroid size	Cys808fs ; WT [1]
30%	Mild TPO deficiency	Subtle elevation of TSH	<u>Gly387Arg</u> ; <u>Gly387Arg</u> [2]
15%		Marked goiter	<u>Trp527Cys</u> ; <u>Trp527Cys</u> †
		High T ₃ to T ₄ molar ratio	<u>Asp224del</u> ; c.820-2A>G †
		High Tg	Gly553C; <u>Asp574</u> <u>Leu575del</u> [3]
0%	Severe TPO deficiency	Marked elevation of TSH	Gln446His; <u>Trp527Cys</u> [4]
		Marked goiter	Cys808fs (homozygous) [1]
		Very high KClO ₄ discharge rate	
		High Tg	

Supplementary Fig. 1 Correlation between clinical phenotypes and *TPO* genotypes

Mutations with residual activity are underlined. Previously, it has been known that biallelic *TPO* mutation carriers show the severe TPO deficiency phenotype (markedly high TSH, marked goiter, high KClO₄ discharge rate and high Tg), while monoallelic mutation carriers and mutation non-carriers have normal thyroid function and morphology. Here, we define a new subtype of TPO deficiency, mild TPO deficiency, which presents subtle elevation of serum TSH, marked goiter, high T₃ to T₄ molar ratio and high Tg. Abbreviations: Tg thyroglobulin; WT, wildtype. †Present report

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