A novel mutation in the human thyroid peroxidase gene resulting in a total iodide organification defect

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Abstract

In this study we describe a novel mutation of the thyroid peroxidase (TPO) gene that resulted in a total iodide organification defect. TPO activity and thyroxine formation in thyroglobulin in the thyroid gland of the patient were below the limits of detection. However, TPO mRNA was detectable at a similar size and concentration as compared with normal thyroid tissues when measured by Northern blot analysis. Sequence analysis of the TPO gene showed the presence of two mutations, a missense mutation in exon 7 and C insertion in exon 14. These mutations were heterozygous and located in different alleles. The latter mutation has already been reported as

one of the mutations of the TPO gene resulting in total iodide organification defect. The former mutation was further analysed by mRNA transfection studies in which mutated mRNA was transfected to CHO-K1 cells by electroporation. The results of transfection studies showed that the cells transfected with mutated mRNA expressed similar size TPO molecules to those of cells transfected with wild-type mRNA but that they lacked TPO activity. The two mutations of the TPO gene resulting in the total iodide organification defect in the patient cosegregated from her parents.

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Introduction

Thyroid peroxidase (TPO) is a key enzyme in the synthesis of thyroid hormones. It catalyzes both iodination and coupling of iodotyrosine residues in thyroglobulin (Tg) (Taurog 1986). The human TPO gene is located on chromosome 2p25 (Endo *et al.* 1995), covers approximately 150 kilobases (kb) of DNA, and is divided into 17 exons (Kimura *et al.* 1989). Full-length TPO mRNA is 3 kb (Kimura *et al.* 1987, Libert *et al.* 1987, Seto *et al.* 1987).

Most cases of congenital hypothyroidism result from dysembryogenesis of the thyroid gland. Hereditary inborn errors in thyroid hormone synthesis are found in 10–20% of these patients, and are mostly caused by defects in the synthesis or iodination of Tg (Bikker *et al.* 1994). The most prevalent cause of inherited defects in thyroid hormone synthesis is believed to be due to TPO deficiency (Manglabruks *et al.* 1991). Recent studies have found seven mutations in the human TPO gene that cause total iodide organification defect (TIOD): 20 bp duplication at position 141 in exon 2 (Bikker *et al.* 1994), GGCC duplication at position 1277 in exon 8 (Abramowicz *et al.* 1992), T1447G missense mutation in exon 9 (Bikker *et al.* 1995), C1708T missense mutation in exon 10 (Bikker

et al. 1996), G1858A missense mutation in exon 10 (Bikker *et al.* 1996), G2485A missense mutation in exon 14 (Bikker *et al.* 1995) and C insertion at position 2505–2511 in exon 14 (Bikker *et al.* 1995). In this paper we describe a novel mutation causing TPO deficiency. This mutation is a missense mutation in exon 7.

Subjects and Methods

Patient

The patient was born after a 41-week gestation in 1977, when neonatal screening for congenital hypothyroidism had not been introduced in Japan. Body weight at birth was 4150 g. At the age of 3·5 months, congenital hypothyroidism was diagnosed on the basis of clinical symptoms suggesting hypothyroidism and laboratory serum data (thyroid-stimulating hormone (TSH), 340 µU/ml (normal range, 0·5–5·0 µU/ml); total thyroxine (T₄), <6·4 nmol/l (normal range, 59·2–162·2 nmol/l); total tri-iodothyronine (T₃), 0·3 nmol/l (normal range, 1·2–3·4 nmol/l); triosorb test, 21·9% (normal range, 25–37%)). Goitre was palpable but not visible.

¹³¹I-Thyroid uptake was 15% at 24 h (normal range, 10–40%). The perchlorate discharge test was positive

(52.9%) 60 min after oral administration of sodium perchlorate at a dose of 1 g, when ^{131}I -thyroid uptake was 10% at 5 h. Serum TSH exhibited an excessively high and delayed response to thyrotrophin (TRH) stimulation. TSH was 240 $\mu\text{U}/\text{ml}$ before, 320 $\mu\text{U}/\text{ml}$ at 30 min, 255 $\mu\text{U}/\text{ml}$ at 60 min, 320 $\mu\text{U}/\text{ml}$ at 90 min and 195 $\mu\text{U}/\text{ml}$ at 120 min after the intramuscular injection of TRH at a dose of 5 $\mu\text{g}/\text{kg}$. The patient was treated with thyroid hormone immediately after admission. At 1.7 years of age, the medication was interrupted transiently and these tests were repeated. ^{131}I -Thyroid uptake was 26% at 3 h and 8% at 24 h. The perchlorate discharge test was positive (82.3%) 60 min after oral administration of sodium perchlorate at a dose of 2 g, when ^{131}I -thyroid uptake was 31% at 4 h. From these results, the patient was diagnosed as TIOD.

During thyroid hormone replacement therapy, small nodular goitres developed in the right lobe at the age of 14 years. The serum Tg level was very high (1050 ng/ml), but anti-Tg autoantibody was not detected. At the age of 18 years, the nodular goitres increased in number and grew. The maximum one was 3×4 cm in diameter. Right lobectomy was performed. The adenoma and surrounding thyroid tissues were frozen separately in liquid nitrogen and stored at -70°C . The pathological finding was follicular adenoma (embryonal type). Preoperative values at the age of 18 years were as follows: TSH, 5.5 $\mu\text{U}/\text{ml}$; total T_4 , 97.8 nmol/l; total T_3 , 1.7 nmol/l; Tg, 3000 ng/ml.

The parents were not consanguineous and had no common ancestors. They had normal thyroid function. Informed consent for these examinations was obtained from the parents because of the patient's low IQ.

Microsomal and supernatant fractions of thyroid tissue

The procedure used to prepare microsomal and supernatant fractions of thyroid tissue has been described previously (Nakagawa *et al.* 1985b). The protein concentration of both fractions was determined by the Bradford method (Bradford 1976) using bovine serum albumin as a standard.

SDS-polyacrylamide gel electrophoresis (PAGE)

The procedure for SDS-PAGE was carried out as described by Laemmli (1970). The supernatant of thyroid tissue (20 μg) was treated with 5% 2-mercaptoethanol and subjected to electrophoresis. Protein bands were visualized by staining with Coomassie brilliant blue R-250.

Western blot analysis

The Western blot procedure has been described previously (Nakagawa *et al.* 1985a). The microsomal fraction of thyroid tissue (20 μg) was electrophoresed on 7.5%

SDS-polyacrylamide gel and transferred to Immobilon-P (Millipore Corporation, Bedford, MA, USA). The membrane was incubated with polyclonal rabbit anti-human TPO antibody (Kotani *et al.* 1986a) and then reacted with goat anti-rabbit IgG antibody conjugated with alkaline phosphatase (Promega Corporation, Madison, WI, USA).

Immunohistochemical staining

Frozen tissue sections (4 μm thick) were stained by an immunoperoxidase streptavidin-biotin complex method using a Histofine SAB-PO(M) kit (Nichirei, Tokyo, Japan). All staining steps were carried out according to the manufacturer's instructions. Mouse monoclonal antibody 16.3.2 (Kotani *et al.* 1986b) was used to stain T_4 .

Guaiacol oxidation assay

TPO activity was determined as described previously (Nakagawa *et al.* 1981). One unit of enzyme oxidized 1 μmol guaiacol/min in 0.1 M phosphate buffer (pH 7.5).

Northern blot analysis

Total cellular RNA was isolated from the adenoma and surrounding thyroid tissues (Kimura *et al.* 1987, Sambrook *et al.* 1989). RNA (10 μg) was electrophoresed through formaldehyde agarose gel, transferred to a Zeta-probe membrane (Bio-Rad, Richmond, NJ, USA), and baked at 80°C for 2 h. The membrane was prehybridized for 3 h, hybridized overnight, and washed according to the manufacturer's instructions. The membrane was exposed to Kodak X-OMAT AR X-ray film with intensifying screens at -80°C . The cDNA probes, a 2.4 kb EcoRI/SacI fragment of human TPO cDNA (Kimura *et al.* 1987) and a 0.4 kb EcoRI fragment of human β -actin cDNA (kindly provided by Dr S Tohda, Tokyo Medical and Dental University, Tokyo, Japan), were labelled with [α - ^{32}P]dCTP using a Takara Random Primer DNA Labelling Kit (Takara Shuzo, Otsu, Japan).

Polymerase chain reaction (PCR)

cDNA was synthesized for reverse transcriptase (RT)-PCR. The reaction mixture (100 μl) contained 5 μg total cellular RNA in 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl_2 , 50 mM KCl, 1 mM of each dNTP, 600 pmol random primer (6 mer; Takara Shuzo), 55 U RNase inhibitor (Takara Shuzo), and 1000 U Moloney murine leukemia virus RT (Gibco BRL, Gaithersburg, MD, USA). The mixture was incubated at 25°C for 10 min, then at 42°C for 60 min and heated at 95°C for 5 min. Using the synthesized cDNA, a reaction mixture (20 μl) for PCR was prepared containing $1 \times$ Ex Taq buffer (Takara Shuzo), 0.5 U Ex Taq (Takara Shuzo), and 4 μl cDNA. The PCR reaction was performed in a Takara

Table 1 PCR primers used in this study

	Primer	Sequence	PCR product
Amplifying region*			
A 24–568	F0024	5'-CAGAAGAGTTACAGCCGTGA-3'	545 bp
of cDNA	R0549	5'-TGTTGCAAGCTCCTGTGATG-3'	
B 519–1058	F0519	5'-AAACACTTGCCTGGCGAACA-3'	540 bp
of cDNA	R1039	5'-AACGAGGTCAACCCGTTTCAT-3'	
C 999–1547	F0999	5'-GCTCTTTGGGAACCTGTCCA-3'	549 bp
of cDNA	R1528	5'-GAGAACACGTTGGACACAGT-3'	
D 1497–2073	F1497	5'-CTATGAAGGCTATGACTCCA-3'	577 bp
of cDNA	R2054	5'-CATCTGCTTCCCAATGAGAC-3'	
E 1975–2546	F1975	5'-CCTGACAACATCGATGTCTG-3'	572 bp
of cDNA	R2527	5'-CCTTTGGTGTTCCTGCACCT-3'	
F 2471–3045	F2471	5'-GCAAAGATGTGAACGAGTGT-3'	575 bp
of cDNA	R3026	5'-CCATGCCTGAGAGTAACAC-3'	
Exon 1	E × 1F	5'-GCAAGTGTCAACAAGTCTGGA-3'	516 bp
	E × 1R	5'-ACTCATCAGTCAGTCAGTCA-3'	
Exon 7	E × 7F	5'-AGGTCATCTTCTGTACCA-3'	389 bp
	E × 7R	5'-CTACCCCTGGGAATAGGACA-3'	
Exon 14	E × 14F	5'-GACCTCCCAGAGAGAAGCA-3'	327 bp
	E × 14R	5'-GATGGTGATTGACAGTTGCC-3'	

*Numbering is according to Kimura *et al.* (1989). F, forward; R, reverse; E, exon.

PCR Thermal Cycler MP (Takara Shuzo) for 30 cycles, each consisting of denaturation at 98 °C for 20 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min.

For genomic DNA PCR, 200 ng genomic DNA prepared from surrounding thyroid tissue or peripheral blood mononuclear cells was used in the reaction mixture instead of 4 µl cDNA. The PCR reaction was carried out using the same procedure as described for RT-PCR. Primer sets used for PCR amplification are summarized in Table 1.

Sequencing

PCR product was directly ligated to the pGEM-T Easy Vector System (Promega Corporation) according to the manufacturer's instructions. Nucleotide sequencing was carried out with an ABI PRISM Dye Primer Cycle Sequencing FS Core Kit (Perkin Elmer, Norwalk, CT, USA) and an ABI 373A automatic sequencer (Perkin Elmer). At least five independent clones of the PCR product were sequenced.

Preparation of mRNA and transfection

PCR product with G808A mutation (primers F0519 and R2527) ligated to the pGEM-T vector and hTPO-1 cDNA inserted to the EcoRI site of the pUC19 vector were cut with BbeI and MluI. Liberated BbeI/MluI fragment from the PCR product was ligated to the BbeI and MluI sites of the hTPO-1 cDNA (G808A cDNA). G808A and hTPO-1 cDNAs cut out from the pUC19 vectors at the sites of EcoRI were blunt-ended and then ligated to the SmaI site of the pSFV-1 vectors (Gibco

BRL, Rockville, MD, USA). Direction of the inserted cDNAs was confirmed by restriction enzyme sites. The pSFV-1 vectors containing G808A and hTPO-1 cDNAs were linearized with SpeI. RNA synthesis was performed according to the manufacturer's instructions using an mMESSAGE mMACHINE Kit (Ambion, Inc., Austin, TX, USA).

CHO-K1 cells were maintained in α -MEM (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% fetal calf serum (FCS) and kanamycin (200 mg/l). Seven million cells and 30 µg mRNA were mixed in a 4 mm cuvette and electroschocked. Electroporation was performed at 260 V, 1500 microfarads and 0.48 µs. Thereafter, the cells were plated in a 10 cm dish and cultured for 24 or 34 h in the presence of 1 µg/ml hematin.

The cells detached with mild trypsin treatment were washed three times with cold phosphate-buffered saline (PBS) and suspended in cold sucrose solution (10 mM K₂HPO₄, pH 7.4, and 0.86% sucrose) at 1×10^7 cells/ml. After brief sonication, a microsomal fraction was prepared and dissolved in 20 mM Tris buffer (pH 7.5). The protein concentration was 5–7 mg/ml.

Results and Discussion

TPO lacking its activity

In both thyroid tissues of the patient, adenoma and surrounding thyroid tissue, Tg was analysed by SDS-PAGE and immunohistochemistry. The results suggested that Tg was normally synthesized but T₄ formation in the Tg molecule was impaired.

Figure 1 Northern blot analysis. (A) 10 µg total cellular RNA from surrounding thyroid tissue (lane 1), adenoma (lane 2), normal thyroid tissues (lanes 3 and 4) and Graves' disease thyroid tissues (lanes 5 and 6) were hybridized to a 2.4 kb TPO cDNA probe. (B) The same filter as used in (A) was rehybridized to a 0.4 kb human β-actin cDNA probe.

TPO enzyme activity was undetectable in the microsomal fractions of the patient's thyroid tissues (normal values, 0.15–0.24 units/mg microsomal protein). The supernatants also showed no activity. Western blot analysis using polyclonal rabbit anti-human TPO antibody revealed that the patient's thyroid tissues contained TPO protein of similar size and concentration to that of normal thyroid tissues. However, the microsomal fractions showed

two extra faint bands (about 80 and 90 kDa). Further analysis of TPO mRNA was conducted by Northern blot. The results showed differences neither in size nor concentration between RNAs from normal thyroid and the patient's tissues (Fig. 1). The above studies indicated that the patient's TPO mRNA and protein were expressed similarly to those of normal thyroid tissues but the patient's TPO protein lacked the enzyme activity.

DNA analysis

A mutation of the TPO gene was first suspected as a reason for the loss of activity of the patient's TPO protein. Therefore, the nucleotide sequence of the TPO mRNA was analysed by RT-PCR using the total cellular RNA isolated from surrounding thyroid tissue. Sequencing of the TPO mRNA revealed five mutations of TPO mRNA with varying frequencies (Table 2). To confirm these mutations, PCR products derived from genomic DNA prepared from surrounding thyroid tissue and PCR primers specific for the exons were sequenced. The results showed a G808A missense mutation (exon 7) and C insertion at position 2505–2511 (exon 14) at frequencies of 3/5 and 3/5 respectively (Table 2). Further sequencing of ten independent clones of RT-PCR products using primers F0519 and R3026 (Table 1) revealed that each clone had only one mutation, either in exon 7 or in exon 14.

Nucleotide sequencing of the patient's TPO mRNA and genomic DNA revealed that the TPO gene mutated at positions 808 (exon 7) and 2505–2511 (exon 14) and that these mutations were heterozygous, one presumably being derived from the patient's mother and the other from her father.

The mother's genomic DNA prepared from peripheral blood mononuclear cells was analysed for the two mutations of the TPO gene. Sequencing of PCR products of exons 7 and 14 showed only the mutation in exon 7, at a frequency of 2/5. It thus was confirmed that one of the

Table 2 TPO mutations in the patient. Nucleotide sequencing was carried out using RT-PCR products of total cellular RNA purified from the patient's thyroid tissue. Four mutations obtained in RT-PCR products were confirmed by sequencing of genomic DNA PCR product. At least five independent subclones of PCR products were sequenced and the number of subclones that had mutations in their sequence is shown as a frequency

	Position*	Nucleotide change	Nature of mutation	Frequency	
				mRNA	Genomic DNA
Exon					
1	78	A deletion	(5'-noncoding)	2/5	0/5
1	84–86	GGT deletion	(5'-noncoding)	2/5	0/5
7	808	G → A	Asp → Asn	5/5	3/5
14	2505–2511	C insertion†	Frameshift	5/10	3/5
17	2973	G → C‡	(3'-noncoding)	5/5	NT‡

*Numbering is according to Kimura *et al.* (1989).
†The mutations were described previously (Bikker *et al.* 1995, 1997).
‡Not tested.

Figure 2 Antibody staining of CHO-K1 cells transfected with mRNAs. For anti-human TPO antibody staining, cells incubated for 24 h were suspended in 3% FCS–PBS at 2×10^6 cells/ml. Cell suspension (100 μ l) was cytospinned on a slide glass and fixed with acetone–PBS–formalin solution. Cells were stained by an immunoperoxidase streptavidin–biotin complex method using a Histofine SAB-PO (M) kit (Nichirei). All figures show anti-human TPO antibody stainings: (a) CHO-K1 cells transfected with hTPO-1 mRNA; (b) CHO-K1 cells transfected with G808A mRNA; (c) CHO-K1 cells transfected with pSFV-1 mRNA; (d) CHO-K1 cells transfected without mRNA. Positive staining percentages were (a) 71%, (b) 65%, (c) 2% and (d) 0%.

two mutations in the patient's TPO gene (exon 7) was derived from her mother. However, we could not analyse the TPO gene of the patient's father, as he was unwilling. The C insertion at position 2505–2511 (exon 14) has already been reported as a mutation of the human TPO gene that results in TIOD (Bikker *et al.* 1995, 1997).

G808A mutation leads to the loss of TPO activity

The missense mutation G808A in exon 7 of the patient's TPO gene resulted in an Asp240Asn substitution of the TPO protein. To determine whether this amino acid substitution leads to the loss of TPO activity, mRNAs, hTPO-1, G808A and pSFV-1 were transfected to CHO-K1 cells. After 24-h or 34-h incubation, cells transfected with mRNA were analysed for their TPO activity and TPO protein expression. As shown in Fig. 2 and Table 3, cells transfected with G808A mRNA showed no TPO activity although TPO protein was expressed. On the other hand, cells transfected with hTPO-1 mRNA exhibited both TPO activity and protein. Western blot analysis, further, supported TPO protein expression both in cells transfected with G808A and hTPO-1 mRNAs (Fig. 3).

G808A-mutated mRNA expressed TPO protein but lacked TPO activity.

Comparison of amino acid sequence neighbouring the exon 7 mutation between various peroxidases

The amino acid sequence neighbouring the mutation was compared with those of various peroxidases. As shown in Fig. 4, Asp238, His239 and Asp240 of human TPO are well conserved among all known TPO amino acid sequences, including pig, mouse and rat, and also in human eosinophil peroxidase (EPO), bovine lactoperoxidase (LPO), and human and mouse myeloperoxidases (MPO). Recently, De Gioia *et al.* (1996) have reported that after constructing their three-dimensional models

Table 3 G808A mutation abrogates TPO activity. CHO-K1 cells were transfected with mRNA by electroporation. After 34-h incubation, they were subjected to guaiacol assay and anti-TPO antibody staining. About 200 cells stained with antibody were counted

	Experiment 1		Experiment 2	
	Guaiacol assay (mU/mg)	Anti-TPO antibody staining (%)	Guaiacol assay (mU/mg)	Anti-TPO antibody staining (%)
Transfected mRNA				
hTPO-1	6.2	46	17	50
G808A	ND	50	ND	49
pSFV-1	ND	2	ND	1
—	ND	1	ND	1

ND, not detectable.

mU/mg, munits/mg microsomal protein.

Figure 3 Western blot analysis of CHO-K1 cells transfected with mRNAs. CHO-K1 cells incubated for 24 h were lysed with SDS-PAGE sample buffer (2×10^6 cells/100 μ l) and boiled for 5 min. After centrifugation at 10 000 *g* for 15 min, 0.5 μ l of the resulting sample was separated through 8% SDS-PAGE and transferred to Immobilon-P (Millipore Corporation). The membrane was incubated with polyclonal rabbit anti-human TPO antibody and then reacted with goat anti-rabbit IgG antibody conjugated with alkaline phosphatase (Promega Corporation): lane 1, CHO-K1 cells transfected with hTPO-1 mRNA; lane 2, CHO-K1 cells transfected with G808A mRNA; lane 3, CHO-K1 cells transfected with pSFV-1 mRNA; lane 4, CHO-K1 cells transfected without mRNA; lane 5, recombinant human TPO (100 ng) (Hata *et al.* 1989).

from structurally conserved regions identified by the multisequence alignment program, His226 of human LPO, His221 of human EPO and His261 of human MPO are more likely to act as distal His which is linked to the iron centre of the heme. If so, His239 of human TPO may be distal His, although Kimura & Ikeda-Saito (1988) suggested that in human TPO the distal His may be located in either exon 9 or 10. Furthermore, the proximal His of human TPO may be His494 in exon 9 (Zeng & Fenna 1992, Bikker *et al.* 1997) instead of the prediction of Kimura & Ikeda-Saito (1988) that the proximal His is located in exon 8. If His239 of human TPO is distal His, the Asp240Asn substitution of the patient's TPO protein would seriously interfere with TPO activity, because

Figure 4 Comparison of the amino acid sequence neighbouring the mutation in exon 7 between various peroxidases. Conserved amino acids are enclosed. The amino acid sequence was adopted from the following references: human TPO, Kimura *et al.* (1987), pig TPO, Magnusson *et al.* (1987), mouse TPO, Kotani *et al.* (1993), rat TPO, Derwahl *et al.* (1989), human EPO, Ten *et al.* (1989), bovine LPO, Dull *et al.* (1990), human MPO, Morishita *et al.* (1987), mouse MPO, Venturelli *et al.* (1989). Abbreviations for the amino acid residues are as follows: A, Ala; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

Asp and Asn are acidic and polar neutral amino acids respectively.

Conclusion

Two mutations of the TPO gene that resulted in a complete loss of TPO enzyme activity were demonstrated in our patient and it was considered that these mutations were heterozygous, with one derived from the patient's mother and the other from her father.

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