Natural course of congenital hypothyroidism by dual oxidase 2 mutations from the neonatal period through puberty

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Abstract

Aim: We previously reported that biallelic mutations in dual oxidase 2 (*DUOX2*) cause transient hypothyroidism. Since then, many cases with *DUOX2* mutations have been reported. However, the clinical features and prognosis of individuals with *DUOX2* defects have not been clarified.

Objective: We investigated the prognosis of patients with congenital hypothyroidism (CH) due to *DUOX2* mutations.

Patients: Twenty-five patients were identified by a neonatal screening program and included seven familial cases. Their serum TSH values ranged from 18.9 to 734.6 mU/l. Twenty-two of the patients had low serum free thyroxine (fT₄) levels (0.17–1.1 ng/dl). Twenty-four of the patients were treated with L-thyroxine.

Methods: We analyzed the *DUOX2*, thyroid peroxidase, Na⁺/I[−] symporter, and dual oxidase maturation factor 2 genes of these 25 patients by PCR-amplified direct sequencing. An additional 11 genes were analyzed in 11 of the 25 patients using next-generation sequencing.

Results: All patients had biallelic *DUOX2* mutations, and seven novel alleles were detected. Fourteen of the patients were able to discontinue replacement therapy, and seven were receiving reduced L-thyroxine doses. Normalization of thyroglobulin lagged several years behind the completion of treatment. Two patients showed permanent hypothyroidism. Except for one case of a learning disability, growth and psychomotor development were normal.

Conclusion: The prognosis of Japanese patients with *DUOX2* defects was usually transient CH. Delayed improvement of thyroglobulin indicates that these patients have subclinical hypothyroidism. Hypothyroidism did not recur in patients during the study period (up to 18 years old).

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Introduction

Congenital hypothyroidism (CH) is one of the most common endocrine diseases in infants. The incidence of CH reported from worldwide neonatal screening programs is between 1/2000 and 1/4000 (1, 2), and its etiology is heterogeneous. The most common cause of CH is thyroid dysgenesis (ectopy, athyreosis and orthotopic hypoplasia), which accounts for 75–80% of cases (3). Defects in

thyroxine synthesis account for 15–20% of cases (3). However, the etiology of many cases is unknown because the underlying cause is not clear.

Recently, the mechanisms of thyroid hormone production and the associated genes have been clarified. The genes associated with thyroid hormone production include thyroid peroxidase (*TPO*), thyroglobulin (*TG*),

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sodium iodide symporter (*NIS*), pendrin (*PDS*), dual oxidase 2 (*DUOX2*), dual oxidase maturation factor 2 (*DUOX2*), dual oxidase 1 (*DUOX1*), dual oxidase 1 maturation factor (*DUOX1*), and iodotyrosine deiodinase (*IYD*) (4, 5, 6, 7, 8, 9, 10, 11, 12), and mutations have been detected in numerous undiagnosed cases. Recent reports have shown that a high proportion of CH cases are caused by mutations in hormone-producing enzymes, and *DUOX2* mutations are one of the most frequent causes (13, 14, 15, 16).

DUOX2, which is also known as thyroid oxidase 2 (*THOX2*) (10, 17), spans 6376 nucleotides on chromosome 15 and includes 33 exons. The encoded *DUOX2* protein is a 1,548-amino acid polypeptide that includes a 26-amino acid signal peptide. *DUOX2* is localized to the apical membrane of thyrocytes, and it is involved in the Ca^{2+} /NADPH-dependent generation of H_2O_2 . To synthesize thyroid hormone, TPO requires H_2O_2 to catalyze both the iodination of tyrosine residues and the coupling of iodotyrosine residues of TG (18).

In 2002, Moreno *et al.* (8) demonstrated that *DUOX2* defects cause CH. They found that patients with homozygous *DUOX2* mutations showed permanent CH, and patients with heterozygous mutations showed transient CH. Since then, additional CH cases with underlying *DUOX2* mutations have been reported (19, 20, 21). In 2008, we reported eight cases of transient CH with biallelic *DUOX2* mutations (13). Ohye *et al.* reported that a homozygous missense mutation (p.R1110Q) causes acquired goiterous hypothyroidism (22). Narumi *et al.* showed that *DUOX2* mutation is the most prevalent cause of CH due to dysmorphogenesis (14). However, many questions remain regarding *DUOX2* mutations, the genetic mechanisms, and clinical course of CH. In this study, we examined 25 Japanese patients with CH caused by biallelic *DUOX2* mutations including follow-ups until up to 18 years of age.

Patients and methods

Patients

Twenty-five patients with biallelic *DUOX2* mutations were included in the study. Twenty-one patients were female, and four were male. Their present ages range from 3 to 18 years. There were seven familial cases (family 1, cases 1–4; family 2, cases 5 and 6; family 3, cases 8 and 9; family 4, cases 10 and 11; family 5, cases 13 and 14; family 6, cases 16 and 17; and family 7, cases 24 and 25), which included two sets of twins (family 4 (monozygotic twins) and family

6 (dizygotic twins)). All familial cases were siblings. All patients were identified based on elevated thyroid stimulating hormone (TSH) levels in a neonatal screening program, and consequently visited our hospital. All the infants had a gestational age >35 weeks (range, 35 weeks 4 days to 41 weeks 3 days; mean, 38 weeks 4 days $\pm$ 11.7 days). Their birth weight and length ranged from 2406 to 3460 g (mean, 2870 $\pm$ 290 g) and 44.0–50.0 cm (mean, 47.4 $\pm$ 1.4 cm), respectively. The observed birth weights and lengths were appropriate for the gestational ages of the patients (Table 1). The TSH concentration at neonatal screening ranged from 10.0 to 242.8 $\mu\text{U/ml}$ (median, 32.75 $\mu\text{U/ml}$). Serum concentrations of TSH, free tri-iodothyronine (fT_3) and free T_4 (fT_4) were 25.7–771.0 $\mu\text{U/ml}$ (median, 94.0 $\mu\text{U/ml}$), 1.89–5.52 pg/ml (median, 3.72 pg/ml) and 0.18–1.50 ng/dl (median, 0.55 ng/dl), respectively (Table 1). TG concentrations were very high, and in most cases, >800 ng/ml, except in two patients (family 7, patients 24 and 25, with 150 and 91.8 ng/ml, respectively). Using a checklist for clinical diagnosis based on the mass-screening guidelines for CH of the Japan Society of Pediatric Endocrinology and Japan Society of Mass Screening, 12 of 23 patients scored above two points for symptoms of CH (e.g., prolonged jaundice, constipation, umbilical herniation, failure to thrive, dry skin, poor activity, macroglossia, hoarseness, cold extremities, edema, opened posterior fontanelles and goiter; more than two points indicates severe CH). Thyroid ultrasonography showed enlargement of the thyroid gland in 11 of the 21 patients. The other ten patients had an orthotopic thyroid gland without enlargement. Six of the 25 patients showed an absence of distal femoral epiphysis (Table 1).

Except for one patient with transient hyperthyrotropinemia in family 1, all patients were treated with L-thyroxine. The first doses of L-thyroxine were determined by the severity of hypothyroidism (check list, elevation of TSH level and reduction of fT_4 level) at first visit to our outpatient department. The usual dose is 5–10 $\mu\text{g/kg}$ per day. However, case 6 did not show apparent reduction in serum fT_3 and fT_4 levels; a lower dose was used.

The reevaluation and classification of patients as having transient or permanent CH were based on normalization of TSH levels despite withdrawal of L-thyroxine. After withdrawal of therapy, serum TSH, fT_3 , fT_4 and TG levels of all patients were followed once or twice a year until now (Table 2).

This study was approved by the ethics committee of Shiga University of Medical Science.

Table 1 Clinical features at neonatal screening and mutations of the dual oxidase 2 gene (*DUOX2*).

Case	Family	Sex	Age	At birth		Height (cm)	Screening		First visit	Femoral		L-Thyroxine (µg/kg per day)	Mutation									
				Gestational age	Birth weight (g)		TSH (µU/ml)	Day		Weight (g)	CL		Goiter	Epiphysis (mm)	TSH (µU/ml)	FT ₃ (pg/ml)	FT ₄ (ng/dl)	TG (ng/dl)	Allele 1		Allele 2	
																			Nucleotide	Amino acid	Nucleotide	Amino acid
1	1	F	18y0m	39w0d	3376	48	36.9	16	3540	1	+	5×6	95.4	2.59	0.43	ND	7	c.1435_1440delCTATC-CinsAG	p.L4795fsX2	c.1883delA	p.K628RfsX10	
2	1	F	16y3m	37w0d	3032	48.5	177.8	28	4030	3	+	5×3	233	1.89	0.19	>800	7.5	c.1435_1440delCTATC-CinsAG	p.L4795fsX2	c.1883delA	p.K628RfsX10	
3	1	F	14y1m	37w6d	3298	49	18.5	25	3298	1	ND	5×3	119	3.32	0.53	>800	5	c.1435_1440delCTATC-CinsAG	p.L4795fsX2	c.1883delA	p.K628RfsX10	
4	1	M	9y3m	37w1d	2854	48.5	10	28	3535	0	ND	4×6	25.7	2.7	1.5	ND	–	c.1435_1440delCTATC-CinsAG	p.L4795fsX2	c.1883delA	p.K628RfsX10	
5	2	F	11y5m	38w2d	2920	48	23.7	24	3980	1	–	4×6	41.9	5.52	0.84	>800	5	c.1588A>T	p.K530X	c.[2635G>A:3200T>C]	p.[E879K:L1067S]	
6	2	F	7y8m	40w1d	3460	50	18.9	15	3950	0	ND	6×3	18.9	4.3	1.33	ND	2.5	c.1588A>T	p.K530X	c.[2635G>A:3200T>C]	p.[E879K:L1067S]	
7		F	7y10m	41w3d	2870	47	29.4	28	4052	0	+	8×6	24.88	4.1	0.62	>800	6.25	c.1946C>A	p.A649E	c.2654G>A	p.R885Q	
8 ^a	3	M	11y7m	40w2d	2740	49	58.3	28	3790	0	–	7×5	114	3.31	0.3	>800	4	c.2033A>G	p.H678R	c.4475G>A	p.R1492H	
9	3	M	13y0m	39w6d	2700	48	71.8	25	3430	0	–	6×4	59.4	4.1	0.58	>800	4.5	c.2033A>G	p.H678R	c.4475G>A	p.R1492H	
10 ^b	4 ^b	F	9y2m	38w4d	2712	47	159.6	15	2865	4	+	0×0	363.89	3.3	0.32	>800	10	c.2654G>A	p.R885Q	c.4027C>T	p.L1343F	
11 ^a	4 ^b	F	9y2m	38w4d	2516	45	210	15	2690	4	+	0×0	406.28	3.2	0.34	>800	10	c.2654G>A	p.R885Q	c.4027C>T	p.L1343F	
12		F	9y6m	37w5d	2626	48	24.4	22	3270	1	–	4×3	89.54	4.1	0.55	>800	7.5	c.2033A>G	p.H678R	c.3200T>C	p.L1067S	
13 ^a	5	F	12y0m	38w2d	2803	49	33	ND	ND	ND	ND	ND	94	ND	1	ND	8	c.2654G>A	p.R885Q	c.2843delG	p.G948VfsX47	
14 ^a	5	F	10y1m	40w2	2904	50	45.5	24	3790	2	–	4×5	145.24	4.2	0.54	ND	8	c.2654G>A	p.R885Q	c.2843delG	p.G948VfsX47	
15		F	12y0M	40w4d	2710	47.3	16.7	22	3360	0	–	7×5	41.95	4	0.62	>800	6	c.[2033A>G:3200T>C]	p.[H678R:L1067S]	c.3478_3480delCTG	p.L1160del	
16 ^c	6 ^c	F	5y10m	35w4d	2656	45	139.6	10	2579	4	+	0×0	240	2.7	0.39	>800	8	c.2033A>G	p.H678R	c.[343G>T:413C>T]	p.[D115Y:P138L]	
17 ^a	6 ^c	F	5y10m	35w4d	2406	44	96.5	10	2350	4	+	0×0	27.38	4.9	1.48	>800	7	c.2033A>G	p.H678R	c.[343G>T:413C>T]	p.[D115Y:P138L]	
18		F	7y0m	39w0d	2750	47	30.6	14	2896	3	+	6×4	68.4	4.3	0.93	>800	8	c.804delG	p.R2685fsX51	c.3478_3480delCTG	p.L1160del	
19		F	5y4m	36w3d	2526	47.5	32.5	13	2636	3	+	4×3	89.54	4.1	0.55	>800	8	c.596delC	p.S199WfsX121	c.1588A>T	p.K530X	
20 ^a		F	15y7m	40w1d	3338	48	26.7	41	4280	2	+	7×5	99.7	3.44	0.54	>800	5	c.2033A>G	p.H678R	c.3096G>C	p.K1032N	
21 ^a		M	3y9m	37w	2900	48.4	19.2	29	4210	2	–	0×0	31.53	4.9	1.01	>800	6	c.2033A>G	p.H678R	c.2335G>A	p.V779M	
22		F	8y0m	41w3d	3230	49	12.9	24	ND	1	–	OK	38.3	4.9	0.81	628	5	c.413C>T	p.P138L	p.3239T>C	p.I1080T	
23 ^a		F	4y8m	39w5d	2610	47.6	242.8	11	2966	5	+	0×0	770.96	2.3	0.18	>800	10	c.2033A>G	p.H678R	c.3329G>A	p.R1110Q	
24	7	F	5y1m	38w0d	2766	48	ND	10	ND	ND	–	2×2	734.61	1.74	0.4	150	10	c.2033A>G	p.H678R	p.3239T>C	p.I1080T	
25 ^a	7	F	3y5m	38w0d	2988	48	89	11	2884	4	–	4×2	648.58	1.7	0.17	91.8	10	c.2033A>G	p.H678R	p.3239T>C	p.I1080T	

ND, no data; CL, check list; TSH, thyroid stimulating hormone; fT₃, free T₃; fT₄, free T₄; TG, thyroglobulin.

^aSubjected to mutation screening by the next-generation sequencing.

^bMonozygotic twin.

^cDizygotic twin.

Table 2 Prognosis in the patients with biallelic mutations of DUOX2.

Case	Family	Age	Normalization of thyroid function (months of age)	Withdrawal T4 therapy (age)	Normalization of TG (age)	Recent data		TSH (μU/ml)	fT ₃ (pg/ml)	fT ₄ (ng/dl)	TG (ng/dl)	Development
						Height (cm)	S.D.					
1	1	18y0m	2	9y4m	13y0m	157.9	-0.03	1.12	2.1	0.9	18.6	Normal
2	1	16y3m	2	8y7m	12y3m	160	0.46	1.49	2.7	1.22	31.6	Normal
3	1	14y1m	2	8y6m	9y7m	150.6	-1.04	1.99	3	1.18	22.7	Normal
4	1	9y3m	1	-	not yet	124.6	-0.8	2.01	3.5	1.35	33.3	Normal
5	2	11y5m	1	3y7m	11y5m	142.4	-0.61	2.09	3.6	1.08	31.1	LD ^a
6	2	7y8m	2	2y7m	not yet	120.8	-0.38	1.44	2.9	1.25	50.1	Normal
7		7y10m	2	7y1m	not yet	120.1	0.66	1.78	4	1.2	66.7	Normal
8	3	11y7m	1	6y1m	not yet	137.5	-1.17	3.77	3.3	0.99	74.3	Normal
9	3	13y0m	1	2y1m	not yet	144.5	-1.58	2.46	3.6	1.14	90.7	Normal
10	4	9y2m	1	40 μg/day	not yet	132.2	0.12	14.32	3.6	1.28	59.9	Normal
11	4	9y2m	1	40 μg/day	not yet	129.2	-0.38	11.48	3.8	1.14	51	Normal
12		9y6m	3	3y9m	9y6m	130.2	-0.53	1.54	3.5	1.04	24.8	Normal
13	5	12y0m	ND	7y4m	12y0m	152.9	0.58	1.29	3.2	1.22	31.2	Normal
14	5	10y1m	2	6y7m	not yet	139.8	0.35	1.33	3.9	1.36	86.7	Normal
15		12y0m	1	7y1m	10y5m	154.9	0.84	2.12	3.4	0.93	27.2	Normal
16	6	5y10m	1	5y10m	not yet	111.5	0.07	2.13	3.1	1.15	46.7	Normal
17	6	5y10m	1	Under reducing	not yet	113.5	0.53	2.64	3.1	1.26	56.6	Normal
18		7y0m	2	Under reducing	not yet	119.1	0.57	6.36	4.1	1.12	161	Normal
19		5y4m	2	Under reducing	not yet	110.5	-0.02	2.86	3.4	1.49	90.3	Normal
20		15y7m	2	2y9m	12y3m	158	0	1.8	3	1.08	5.9	Normal
21		3y9m	3	30 μg/day	not yet	103.5	1.32	7.81	3.1	1.49	37.3	Normal
22		8y0m	2	Under reducing	not yet	127.1	0.46	3.63	4.8	1.96	47.6	Normal
23		4y8m	2	Under reducing	3y9m	120.1	0.66	1.27	4.1	1.48	16.1	Normal
24	7	5y1m	1	Under reducing	5y1m	104.8	-0.44	1.05	4.54	1.83	17.7	Normal
25	7	3y5m	1	Under reducing	not yet	89.8	-1.34	0.14	5.05	2.01	95.2	Normal

ND, no data.

^aLD: mild learning disability.

Laboratory testing and sequence analysis

At neonatal screening, TSH was measured by the Cretin TSH ELISA II assay (Eiken Chemical, Tokyo, Japan). During hospital visits, TSH was measured using a fluorescent enzyme immunoassay method (TOSHO, Tokyo, Japan). fT_4 and fT_3 levels were measured with a fluorescent enzyme immunoassay (TOSHO). TG was measured with an immunoradiometric assay (Eiken Chemical).

Genomic DNA was extracted from blood leukocytes using standard techniques after obtaining informed consent from patients and their families to participate in this study. Amplification of exons and exon-intron boundaries by PCR from genomic DNA was performed using the ten pairs of oligonucleotide primers shown in [Supplementary Table 1](#), see section on [supplementary data](#) given at the end of this article (13). The genomic DNA sequences and cDNA sequences were based on those in the human genome database (NCBI accession numbers CCDS10117.1 and AF267981). The 33 exons of *DUOX2* were amplified as ten PCR products. PCR products were purified using the Nucleospin Gel & PCR Clean-up Kit (TaKaRa, Kyoto, Japan), and the sequences of the amplified DNA fragments were determined directly using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems) and an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems). The sequencing primers are shown in [Supplementary Table 1](#).

The amplified *DUOX2* fragments were subcloned into the pCR 2.1 vector using the TA Cloning Kit (Invitrogen) to verify the mutant sequence in patients with deletion or insertion mutations, and to determine the *cis* or *trans* arrangement of the two mutations in one exon in cases 16 and 17. Each PCR product (30 ng), including the regions harboring mutations, was ligated with the vector (50 ng) and transformed into cells using the Competent High JM109 Kit (Toyobo, Osaka, Japan). The *DUOX2* sequences of the patients' relatives were analyzed after obtaining informed consent. *Cis* or *trans* arrangement of the multiple variations was determined by analyzing the *DUOX2* genes of the patients' parents.

To analyze the genes encoding *TPO*, *NIS* and *DUOXA2*, all coding exons and surrounding intronic sequences were amplified by PCR and were sequenced directly as described previously (4, 6, 9, 13).

Eleven of the 25 patients (8, 10, 11, 13, 14, 16, 17, 20, 21, 23, and 25), who were enrolled in the study after July 2012, were subjected to mutation screening for *DUOX1*, *DUOX2*, *DUOXA1*, *DUOXA2*, *GNAS*, *FOXE1*, *IYD*, *NIS*, *NKX2-1*, *NKX2-5*, *PDS*, *PAX8*, *TG*, *TPO* and *TSHR* using the

Haloplex Target Enrichment System (Agilent Technologies, Palo Alto, CA, USA). The library was sequenced as 150-bp paired-end reads on a MiSeq next-generation sequencer (Illumina, San Diego, CA, USA). Nucleotide alterations were called by the Surecall system (Agilent Technologies) and SAMtools 0.1.17 software (<http://samtools.sourceforge.net>) and were confirmed by Sanger direct sequencing.

The functional role of seven *DUOX2* variants (p.D115Y, p.A649E, p.V779M, p.E879K, p.R885Q, p.L1343F, and p.R1492H) and three polymorphisms (p.L138L, p.H678R, and p.L1067S) were evaluated by using three different bioinformatics programs (PolyPhen-2 Polymorphism Phenotyping v2 (<http://genetics.bwh.harvard.edu/pph2/>), Mutation Tester (<http://www.mutationtaster.org>) and SIFT (<http://sift.jcvi.org>)), which predict the possible impact of an amino acid substitution on the structure and function of human proteins using straightforward physical and evolutionary comparative considerations.

Results

Identification of variants by *DUOX2* sequencing

All patients had biallelic *DUOX2* variants. Twenty-one distinct variants were detected ([Table 1](#)). The novel variants are shown in [Supplementary Figures 1, 2, 3, 4, 5, 6 and 7](#), see section on [supplementary data](#) given at the end of this article. Variants p.V779M (rs145061993) and p.R1492H (rs147945181 and rs144543420) were previously reported in SNP databases. These novel seven variants have not been reported as polymorphisms in the Japanese population. The allelic frequencies of these variants in Japanese individuals are likely to be <0.01 (Human Genetic Variation database, <http://www.genome.med.kyoto-u.ac.jp/SnpDB/>). The most prevalent allele was c.2033A>G (p.H678R); 10 of the 25 patients had this allele ([Fig. 1](#)).

None of the patients had biallelic mutations in any other genes involved in the generation of thyroid hormone (*TPO*, *NIS*, and *DUOXA2*). Furthermore, the 11 patients analyzed by next-generation sequencing only had biallelic mutations in *DUOX2*. Cases 10 and 11 (twins) also had a monoallelic mutation in TG (p.T1498M). Cases 16 and 17 (twins) had a heterozygous mutation in TG (p.R2585W). Both TG variants were reported on the web as rare and probably damaging variants by PolyPhen-2 (<http://evidence.gpg-hms.org/TG-T1498M>, <http://evidence.gpg-hms.org/TG-R2585W>). All the parents of the patients

Figure 1

The 21 mutations detected in this study are shown on a topological model of the DUOX2 protein, which was based on structural data from De Deken *et al.*, Vigone *et al.* and Maruo *et al.* (10, 13, 19). The locations of all DUOX2 mutations reported to date (8, 13, 14, 15, 16, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28) are shown as small closed circles indicated by arrows with small characters. The locations of the 21 mutations detected in

this study are shown as open boxes with large characters. The seven novel mutations found in the present work are shown as large open circles. The dotted lines indicate the linkages between the mutations. The relative positions of the calcium-binding (EF-hand), flavin adenine dinucleotide (FAD)-binding and NADPH-binding motifs are also indicated (10, 17).

included in this study are carriers of monoallelic DUOX2 mutations. They did not have a history of hypothyroidism.

Prediction of the functional effects of missense variants in DUOX2 by in silico assays

Prediction of the functional effects of the seven missense variants by in silico assays showed that p.D115Y, p.A649E, p.E879K, p.R885Q and p.R1492H were probably damaging and that p.V779Q, p.I1080T and p.L1343F are possibly

damaging. The results suggested that the seven missense mutations likely have a disease-causing role. In contrast, the three polymorphic variants (p.P138L, p.H678R and p.L1067S) were not predicted to be disease-causing variants in the in silico assays (Table 3).

Clinical course of patients with biallelic mutations in DUOX2

After beginning L-thyroxine treatment, serum TSH, fT₃ and fT₄ levels in most patients improved within 2 months.

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Table 3 Prediction of functional effects of missense variants of *DUOX2* by in silico assays.

Variant	Polyphen2 ^a		Mutation tester	SIFT Human Protein	SIFT Score
p.D115Y	Probably damaging	0.99	Disease causing	Damaging	0
p.A649E	Probably damaging	0.94	Disease causing	Tolerated	0.07
p.V779M	Possibly damaging	0.895	Disease causing	Damaging	0.01
p.E879K	Probably damaging	1.00	Disease causing	Damaging	0.04
p.R885Q	Probably damaging	0.998	Disease causing	Tolerated	0.06
p.I1080T	Possibly damaging	0.834	Disease causing	Damaging	0
p.L1343F	Possibly damaging	0.831	Disease causing	Damaging	0
p.R1492H	Probably damaging	1.00	Disease causing	Damaging	0
p.P138L	Benign	0.031	Polymorphism	Tolerated	0.06
p.H678R	Benign	0.000	Polymorphism	Tolerated	1
p.L1067S	Benign	0.025	No data	Tolerated	0.85

^aThe PolyPhen-2 algorithm calculates the naïve Bayes posterior probability that a given mutation will be damaging and qualitatively predicts that it will be benign, possibly damaging or probably damaging, corresponding to posterior probability intervals (0, 0.2), (0.2, 0.85), and (0.85, 1), respectively.

For most patients, the L-thyroxine dose could be reduced by about 2–4 years of age, except for the twins in cases 10, 11 and 21. Twenty-one of the 24 patients were able to receive reduced doses of L-thyroxine. Fourteen patients ranging in age from 2 years 1 month to 9 years 4 months were able to stop L-thyroxine treatment (median, 8 years 2 months). For the remaining seven patients, L-thyroxine was withdrawn by the conclusion of the study (ages 3–8 years). For the twins (cases 10 and 11) with compound heterozygous mutations p.R885Q and p.L1343F, it was necessary to increase the L-thyroxine dose to control the elevation of TSH and permanent CH. For patient 21, with p.H678R and p.V779M, L-thyroxine also could not yet begin to be withdrawn. Elevated serum TG levels persisted after terminating L-thyroxine treatment. However, TG levels usually normalized after the patients reached 10 years of age (cases 1, 2, 3, 5, 12, 13, 15 and 20; Table 2). This indicates that the patients with *DUOX2* mutations have subclinical hypothyroidism until puberty. After withdrawal of therapy, all patients were followed until recently (for 9 months–13 years) (Table 2). During puberty, none of the transient cases showed sign of hypothyroidism (elevation of TSH or reduction of fT₄ level).

The oldest patient, who was compound heterozygous for p.L479SfsX2 and p.K628RfsX10, showed no signs of hypothyroidism after finishing treatment for the remainder of the study period (i.e., until she reached 18 years of age), and her TG level remained in the normal range.

The growth of the patients was within the normal range, and psychomotor development was also normal except for patient 5, who exhibited a mild learning disability (Table 2).

Discussion

After the discovery of the connection between *DUOX2* deficiency and CH, many patients with *DUOX2* mutations have been reported (Fig. 1) (8, 13, 14, 15, 16, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28). However, some questions still remain. For example, which *DUOX2* mutations result in permanent CH and which result in transient hypothyroidism? What is the natural course of *DUOX2* deficiencies?

Moreno *et al.* (12) reported that biallelic *DUOX2* mutations cause permanent CH and monoallelic mutations cause transient CH. However, there is a manuscript supporting the hypothesis that carriers of monoallelic mutations only suffer from transient CH (29). This recent study also suggested the involvement of a monoallelic variant of *DUOX2* in the development of transient CH. Other reports have shown that biallelic *DUOX2* mutations cause mild to moderate CH (13, 14, 15, 16, 19, 22, 30). Recently, some studies have shown that *DUOX2* defects are the predominant cause of CH associated with dysmorphogenesis (13, 14, 15, 16, 25, 26).

In our analysis of 25 Japanese patients with CH carrying biallelic *DUOX2* mutations, 13 patients finished L-thyroxine replacement therapy prior to reaching 10 years of age, and eight patients, ranging from 3 to 8 years old at the end of the study period, were receiving reduced L-thyroxine doses. This result indicates that in the Japanese population, *DUOX2* mutations can cause transient CH, even combinations of inactivation mutations (i.e., nonsense and frameshift mutations) as observed in family 1 (cases 1, 2, 3, and 4) and case 19. Moreover, case 4 in family 1, with compound heterozygous p.L479SfsX2 and p.K628RfsX10 mutations, showed only transient hyperthyrotropinemia, whereas the patient's three elder

sisters showed apparent transient CH. These results indicate that *DUOX2* mutations cause mild to moderate forms of hypothyroidism, including transient CH and transient hyperthyrotropinemia. A possible mechanism by which *DUOX2* mutations cause transient CH is as follows. Two dual oxidases in thyrocytes, *DUOX1* and *DUOX2*, generate H_2O_2 for organification and coupling of tyrosine (10, 17). However, the expression levels of these two *DUOX*s differ; the *DUOX1* expression level is one-fifth that of *DUOX2* (31). Even if *DUOX2* enzyme activity is lost, a low level of H_2O_2 may be maintained by *DUOX1* throughout life. The requirement for thyroid hormone during the neonatal period (10–15 $\mu\text{g}/\text{kg}$) is 5–7 times higher than that of adults (2 $\mu\text{g}/\text{kg}$) (32, 33); then, the thyroid hormone requirement gradually decreases after the infantile period. The H_2O_2 supply from *DUOX1* alone may be inadequate during the neonatal and infantile periods, so individuals with *DUOX2* deficiencies are liable to show signs of CH. As the thyroid hormone requirement decreases over the course of development, *DUOX1* production of H_2O_2 is sufficient to maintain thyroid hormone synthesis, irrespective of *DUOX2* mutations (Fig. 2). However, the members of Family 1 were not analyzed by next-generation sequencing. The presence of other unidentified defects may be involved in the difference in the severity.

In addition to the frameshift mutations (p.S199WfsX121, p.R268SfsX51 p.L479SfsX2, p.K628RfsX10, and p.G948VfsX47) and the one amino acid deletion variant (p.L1160del), prediction of the functional effect by *in silico* assays suggested that seven of the missense variants – p.D115Y, p.A649E, p.V779M, p.E879K, p.R885Q, p.L1343F, and p.R1492H – were disease-causing variants. In contrast, the *in silico* assays suggested that the three polymorphic variants – p.P138L, p.H678R, and p.L1067S – were silent polymorphisms. This was consistent with a previous expression study (25). Previous studies showed that p.H678R did not reduce H_2O_2 production (14, 34). The most prevalent variant detected in this study was p.H678R. Previously, Narumi *et al.* (14) reported that this variant is a polymorphism in the Japanese population and that it has an interactive effect with other mutations leading to a mild phenotype. Therefore, patients who are compound heterozygous for p.H678R and other pathological variants might exhibit mild clinical manifestations due to *DUOX2* defects. However, even patients with a combination of nonsense and frameshift mutations (i.e., cases 1, 2, 3, 4 and 19) showed transient CH. The location of the mutations within the *DUOX2* protein varied (Fig. 1). Moreover,

Figure 2

The proposed mechanism underlying the development of transient CH due to *DUOX2* defects. Two dual oxidases, *DUOX1* and *DUOX2*, generate H_2O_2 for thyroid hormone production in thyrocytes. The amount of *DUOX1* expressed is one-fifth that of *DUOX2*. If *DUOX2* enzyme activity is lost, a low level of H_2O_2 is maintained by *DUOX1* throughout life. The requirement for thyroid hormone during the neonatal period is 5–7 times higher than it is in adults, and it gradually decreases after the infantile period. The H_2O_2 supply from *DUOX1* alone may be inadequate during the neonatal and infantile periods, so that carriers with *DUOX2* deficiency exhibit hypothyroidism. As the thyroid hormone requirement decreases, the production of H_2O_2 by *DUOX1* is sufficient to maintain thyroid hormone synthesis, irrespective of the *DUOX2* genotype.

some patients (cases 10 and 11; compound heterozygotes p.R885Q and p.L1343F alleles) also exhibited permanent CH. Therefore, it is difficult to detect associations between *DUOX2* mutations and prognosis.

The results of the next generation sequencing of 11 patients, showing no other biallelic mutations in thyroid hormonogenesis genes, indicated that a *DUOX2* defect is an important cause of hypothyroidism. However, modulators other than the genetic polymorphism are thought to exist that lead to the variety observed in the patients' clinical manifestations. Permanent CH cases (cases 10 and 11) had biallelic mutations in *DUOX2* and a monoallelic mutation in *TG*. However, cases 16 and 17 with transient CH had a combination of the biallelic mutations in *DUOX2* and the monoallelic mutation in *TG*. A recent report revealed that the combination of biallelic mutation in *DUOX2* with the monoallelic mutation in a different thyroid hormone-generating enzyme might cause CH. (35). However in some cases, the combination of biallelic mutation in *DUOX2* with the monoallelic mutation in a

different thyroid hormone-generating enzyme might cause permanent CH.

Another factor in patients with DUOX2 deficiencies that might affect the differential prognosis between transient CH in Japanese and permanent CH in Europeans is the iodine content in soil. In Europe, the iodine content in the soil is low, whereas in Japan, it is plentiful (36). This difference might explain the different phenotypes observed in Europe and Japan, as a higher iodine supply might increase thyroid hormone production.

In our study, there was an unbalanced sex ratio, with 21 females and four males. It was not clear why such sex-based differences occurred. However, in family 1 (compound heterozygous for p.L479SfsX2 and p.K628RfsX10), the three elder sisters showed apparent transient hypothyroidism, whereas the younger brother showed only transient hyperthyrotropinemia. These cases suggested that the symptoms of the DUOX2 defect might be different in each individual, even in those with same mutations.

TG levels were elevated for 3–10 years after the L-thyroxine treatments ended (Table 2). After the start of puberty, when the L-thyroxine requirement is reduced to that of adults, the TG level improved to within the normal range. The delayed improvement of the TG level suggests that patients with DUOX2 defects might exhibit insufficient thyroid hormone production. After stopping the L-thyroxine treatment, TG levels improved, and hypothyroidism did not recur in the patients from puberty to 18 years of age (cases 1, 2, 3, 5, and 20). However, Ohye *et al.* showed that a patient who was homozygous for p.R1110Q developed acquired goiterous hypothyroidism (23). The goiter appeared when the patient was in her 20 s, and hypothyroidism developed when the patient was in her 40 s. Abe *et al.* (22) reported a 39-year-old mother with a homozygous mutation (p.H678R) and a mild phenotype that also showed elevated TSH (15.6 μ U/ml). Accordingly, our patients with transient CH might develop hypothyroidism later in life. Given the increased need for thyroid hormone during pregnancy, pregnant women with a history of transient CH due to biallelic mutations in *DUOX2* should be observed carefully, especially after withdrawal of L-thyroxine. Further observations are necessary to clarify the clinical course of DUOX2 defects.

In this study, the growth and development of patients identified by neonatal screening were largely normal. In contrast, a previous report of Turkish patients with homozygous p.R434X showed mental retardation due to delayed diagnosis at 6 months of age (26). Therefore, early diagnosis by neonatal screening programs and early

treatment are important for patients with DUOX2 defects, even for transient CH.

Conclusion

Japanese patients with DUOX2 defects primarily showed transient CH. Many patients completed L-thyroxine replacement therapy before reaching puberty. As some patients showed permanent CH and transient hyperthyrotropinemia, *DUOX2* mutations might be associated with various phenotypes. To clarify the phenotypes and clinical course of *DUOX2* mutations, additional data and patient observations are necessary.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/EJE-15-0959>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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