Homozygous *DUOXA2* mutation (p.Tyr138*) in a girl with congenital hypothyroidism and her apparently unaffected brother: Case report and review of the literature

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Abstract. Mutations in *DUOXA2*, encoding dual oxidase maturation factor 2, is a rare genetic cause of congenital hypothyroidism. Only four biallelic *DUOXA2* mutation carriers have been described to date. This study was conducted to report the clinical and genetic findings of a *DUOXA2* mutation-carrying family, and to review the previously reported cases. The proband was a 4-year-old girl, who was diagnosed as having congenital hypothyroidism in the frame of newborn screening. She had a high serum TSH level (138 mU/L) and a low free T4 level (0.4 ng/dL). Ultrasonography revealed goiter. She was immediately treated with levothyroxine. At age 3 years, reevaluation of her thyroid function showed a slightly elevated serum TSH level (11.0 mU/L) with normal free T4 level. Screening of the eleven congenital hypothyroidism-related genes demonstrated a previously reported nonsense *DUOXA2* mutation (p.Tyr138*) in the homozygous state. Unexpectedly, we also found that the elder brother of the proband, who had no significant past medical history, had the identical homozygous mutation. Using expression experiments with HEK293 cells, we confirmed that p.Tyr138* was a loss-of-function mutation. In the literature, clinical courses of three patients were described, showing characteristic age-dependent improvement of the thyroid function. In conclusion, The proband showed comparable clinical phenotype to previously reported cases, while her brother was unaffected. The phenotypic spectrum of *DUOXA2* mutations could be broader than currently accepted.

Key words: Congenital hypothyroidism, DUOXA2, Mutation, Genetics, Newborn screening

IODINATION of thyroglobulin (Tg) is the crucial step of the thyroid hormone-producing pathway, and it is governed by three types of thyroid-specific molecules: thyroid peroxidase (TPO), dual oxidases (DUOXs; DUOX1 and DUOX2) and DUOX maturation factors (DUOXAs; DUOXA1 and DUOXA2). DUOXAs play indispensable roles to make DUOXs functional proteins [1]. DUOXAs help endoplasmic reticulum-to-Golgi transition, maturation, and translocation to the plasma membrane of DUOXs.

DUOXs, belonging to nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-family molecule, generate H_2O_2 at the apical membrane of the thyroid follicular cells by transferring electrons from intracellular NADPH across the membrane and coupling these to oxygen [1]. The produced H_2O_2 is in turn utilized by TPO to oxidize iodide ions to form iodine atoms for addition onto tyrosine residues of Tg (*i.e.,* Tg iodination).

There are two forms of DUOXs (DUOX1 and DUOX2) [2], and also two forms of DUOXAs (DUOXA1 and DUOXA2) [1]. DUOX2 and DUOXA2 are thought to form the principal H_2O_2 generator in the thyroid, considering the higher mRNA expression levels than their paralogs [1, 3]. The principal role of DUOX2/DUOXA2 in thyroid hormone production is

also evidenced by the facts that both biallelic *DUOX2* mutations [4] and biallelic *DUOXA2* mutations [5] cause congenital hypothyroidism (CH) in humans, while neither *DUOX1* mutations nor *DUOXA1* mutations have been identified so far. Nonetheless, DUOX1 (and possibly DUOXA1) could have a minor role in H_2O_2 production in the thyroid, because CH in biallelic *DUOX2* mutation carriers is often transient [6], and those mutation carriers produce thyroid hormones presumably *via* DUOX1 in the post-infantile period [7]. As for *DUOXA2* mutation carriers, only four unrelated CH patients have been reported [5, 8-10], and thus the phenotypic spectrum remains to be established. Here, we report the fifth *DUOXA2* mutation-carrying CH patients, whose brother also had the identical homozygous mutation but did not have CH.

Materials and Methods

Patient

The patient, a 4-year-old girl, was the second child of healthy unrelated parents that had migrated from Korea to Japan. The course of pregnancy and delivery was uneventful. She was born at term with birth length 49.0 cm and weight 2,912 g. Because she had a positive result in newborn screening for CH (blood-spot TSH level 78 mU/L; cutoff < 15 mU/L), she was referred to us at age 13 days. At this point, she had normal weight gain (28 g/day), and had no hypothyroidism-related manifestation. Blood tests revealed a high serum TSH level (138 mU/L; reference 1.7-9.1), a low free T4 level (0.4 ng/dL; reference 0.9-2.3), a low free T3 level (2.8 pg/mL; reference 3.3-4.9) and a high serum Tg level (> 1,000 ng/mL; reference 3.8-56.3). Urinary iodine level was high (6,540 µg/g·creatinine; reference 71-1,660 µg/g·creatinine) [11]. Distal femoral epiphyses were present. Ultrasonography showed goiter (thyroid size, +5.9 SD) [12]. She was diagnosed as having CH, and levothyroxine supplementation was started at 35 µg/day (10 µg/kg/day). The thyroid function test results were stabilized by the initial dose, and required no further titration. She grew normally and developed satisfactorily after the initiation of the treatment. At age 3 years, we reevaluated her thyroid function with stopping treatment for 4 weeks. She had a slightly elevated serum TSH level (11.0 mU/L), with normal thyroid hormone levels (free T4, 1.2 ng/dL; free T3, 4.3 pg/dL). Levothyroxine supplementation was restarted.

The elder brother of the proband was a 10-year-old boy. He was born in Japan, received TSH-based newborn screening, and the result was negative. He had no significant past medical history. The growth and development were normal. At age 9 years, after the identification of a homozygous *DUOXA2* mutation in him, we evaluated his thyroid functions. He had a normal serum TSH level (2.0 mU/L), a normal free T4 level (1.1 ng/dL) and a normal free T3 level (3.8 pg/dL), but had a high serum Tg level (106 ng/dL; reference <30). He had normal serum total cholesterol levels (181±10 mg/dL, N=5) and normal serum creatinine kinase levels (176±40 U/L, N=5). Ultrasonography showed a slightly enlarged thyroid gland (thyroid size, +1.7 SD). At age 10 years, we performed TRH test, revealing normal basal (2.6 mU/L) and stimulated (17.0 mU/L) serum TSH levels. The serum Tg level was repeatedly high (89 ng/mL).

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 of the proband. Genomic DNA samples were collected from the proband, her brother and parents. Eleven known genes associated with CH (*DUOX2*, *DUOXA2*, *FOXE1*, *IYD, NKX2-1, PAX8, SLC5A5*, *SLC26A4*, *TG*, *TPO* and *TSHR*) and two candidate genes (*DUOX1* and *DUOXA1*) 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 [13]. Base calling, read filtering, and demultiplexing were performed with the standard Illumina processing pipeline. We used BWA 0.7.5 [14] 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 [15] with the default settings. We used ANNOVAR [16] for annotation of called variants. The detected mutation was confirmed by standard PCR-based Sanger sequencing with previously described methods [17].

Functional analyses

Plasmid vectors encoding N-terminal hemagglutinin (HA)-tagged human DUOX2, and human DUOXA2 have been reported previously [17]. A vector encoding the p.Tyr138* mutation was created with the

Gibson assembly technique (NEbuilder; New England Biolabs, Ipswich, MA, USA). Culture and transient transfection of HEK293 cells were performed as previously described [17].

Cell surface expression of HA-DUOX2 were evaluated using cells co-transfected with HA-DUOX2 and each DUOXA2 [wildtype (WT) or Tyr138*] or empty vector. Forty-eight hour after transfection, cells were washed twice with PBS, fixed in 4% paraformaldehyde at room temperature for 10 min, and blocked in 5% bovine serum albumin/PBS at room temperature for 1 h. Then, the cells were incubated with the anti-HA monoclonal antibody (clone 3F10, Roche Life Science, Indianapolis, IN, USA), washed three times with PBS, and incubated with AlexaFluor 555-conjugated anti-rat IgG antibody (Thermo Fisher Scientific, Waltham, MA, USA). Nuclei of cells were stained with Hoechst 33342. The cells were observed with Olympus IX71 fluorescence microscope.

H_2O_2-producing capacity of cells co-expressing HA-DUOX2 and each DUOXA2 (WT or Tyr138*) or empty vector was evaluated with previously described methods using Amplex Red reagent (Thermo Fisher Scientific) [17]. Relative H_2O_2-production was expressed as percentage (mean ± SEM) of WT activity, where basal activity (measured with non-transfected 293 cells) was set to 0%. Data are representative of three independent experiments (each performed in triplicate) with similar results.

Literature review

A review of the articles published prior to 1 October 2016 was conducted independently by two authors (C.S. and S.N.). PubMed and Google Scholar search engines were used to identify relevant articles using keywords "DUOXA2", "dual oxidase maturation factor 2".

Results

Mutation detection

Next generation sequencing-based comprehensive genetic analysis of the proband led us to identify a homozygous nonsense *DUOXA2* mutation (c.413dupA, p.Tyr138*) that had been previously described [9]. No mutation was found in the other sequenced genes. The presence of the mutation was confirmed by Sanger sequencing (Fig. 1A). The mutation causes loss of three transmembrane domains (Fig. 1B), and is highly likely to be a null mutation. Family analysis showed that the parents of the proband had the mutation in the heterozygous state (Fig. 1C). The elder brother of the proband, who is not affected by CH, also had the mutation in the homozygous state.
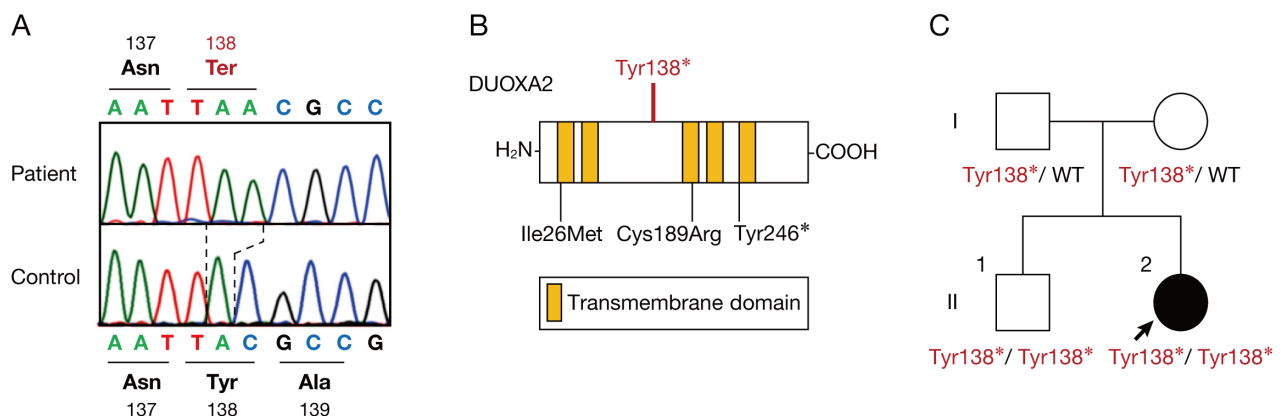

Fig. 1 Identification of a homozygous *DUOXA2* mutation, p.Tyr138*

A, Partial electropherograms of the PCR products of DUOXA2 exon 4 (NM_207581). The patient had a 1-bp duplication of adenine, creating a stop codon (TAA) with altering Tyr138. **B**, A schematic diagram of the DUOXA2 protein. The locations of loss-of-function mutations are also indicated. The p.Tyr138* mutation is predicted to cause loss of three transmembrane domains. **C**, Pedigree of the *DUOXA2* mutation-carrying family. A black symbol represents affection of congenital hypothyroidism. The identical homozygous mutation was observed in the proband (II-2) and her elder brother (II-1), but congenital hypothyroidism was only seen in the proband.

Functional analyses

To verify the pathogenicity of the p.Tyr138* mutation, we conducted functional analyses with HEK293 cells. As previously described by Grasberger and Refetoff [1], the DUOX2 protein could not localize to cell surface without DUOXA2, while coexpression of the DUOXA2 protein gave rise to cell surface expression of DUOX2 (Fig. 2A). The p.Tyr138*-DUOXA2 protein did not have the effect (Fig. 2A), indicating a loss-of-function mutation. Compatible with the results of intracellular localization analysis, cell surface H_2O_2-producing capacity was negligible in cells coexpressing the DUOX2 protein and the p.Tyr138*-DUOXA2 protein (Fig. 2B), again indicating that p.Tyr138* is a loss-of-function mutation.

Literature review

In the literature, four CH patients with biallelic *DUOXA2* mutations were found [5, 8-10], and clinical information was available in three out of the four published cases (Table 1) [5, 8, 10]. Blood-spot TSH levels at newborn screening ranged from 48 to 135 mU/L, and serum TSH levels at the first medical examination ranged from 12 to 151 mU/L. Two patients had low serum total T4 levels (5.2 and 39 µg/dL; reference 106-195), and the remaining one had a low serum free T4 level (0.2 ng/dL). All three patients were shown to have goiter by ultrasonography. They all received levothyroxine replacement therapy. Reevaluation of

thyroid function with stopping therapy was conducted at ages from 2 to 7 years. Mildly elevated serum TSH levels (5.0 to 6.5 mU/L) with normal thyroid hormone levels were shown. Perchlorate discharge test was performed in only one patient showing normal ^{123}I uptake (10.5% at 2 hr; reference 2-12) with a partial perchlorate-induced discharge (18 %; reference <10).

Discussion

All *DUOXA2* mutation carriers reported to date were sporadic CH patients, and none of the family members of the probands had biallelic *DUOXA2* mutations. Therefore, this study reports the first mutation-carrying family in which two members had the identical biallelic mutation. The clinical presentation of the proband was comparable to those of previous reports, including overt hypothyroidism with goiter at first presentation, and subsequent improvement of the thyroid hormone-producing capacity. The common clinical presentation of *DUOXA2* mutation carriers resembles that of *DUOX2* mutation carriers, whose thyroid functions improve in an age-dependent manner [7]. Age-dependent improvement of the *DUOX2* defect is likely explained by two factors: (i) age-dependent decrease of thyroid hormone requirement per body weight, and (ii) compensation of H_2O_2 production by *DUOX1* [7]. We speculate that similar mechanisms are also valid for the
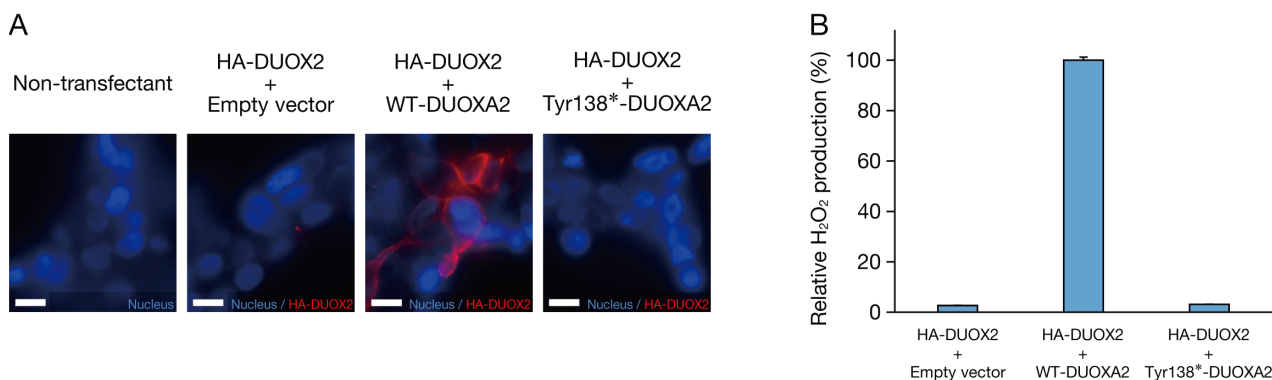

Fig. 2   Functional characterization of the p.Tyr138* *DUOXA2* mutation

A, HEK293 cells were transiently transfected with N-terminal HA-tagged DUOX2 (HA-DUOX2) and DUOXA2 [wildtype (WT) or p.Tyr138*] or empty vector. Immunofluorescence without cell permeabilization was performed, enabling assessment of cell surface expression of HA-DUOX2. Coexpression of WT-DUOXA2 resulted in cell surface expression of HA-DUOX2, while coexpression of p.Tyr138*-DUOXA2 did not. Scale bars indicate 15 µm. **B**, Cell surface H_2O_2 production of cells expressing HA-DUOX2 and DUOXA2 (WT or p.Tyr138*) or empty vector was evaluated with Amplex Red reagent. The H_2O_2-producing capacity was negligible in cells expressing HA-DUOX2 and p.Tyr138* DUOXA2.

Table 1 Clinical phenotypes of the biallelic *DUOXA2* mutation carriers

Author, year	Zamproni *et al.* 2008	Hulur *et al.* 2011	Liu *et al.* 2015	Present report II-2	Present report II-1	Reference range
Sex	Female	Male	Female	Female	Male	
Nationality	Chinese	German	Chinese	Japanese	Japanese	
DUOXA2 mutation	p.Tyr246* (homozygous)	p.Cys189Arg Gene deletion †	p.Tyr246* (homozygous)	p.Tyr138* (homozygous)	p.Tyr138* (homozygous)	
Blood-spot TSH at NS (mU/L)	48	58	135	78	Normal	
Serum TSH at first visit (mU/L)	12	151	129	138	NA	<9.1
Thyroid ultrasonography	Goiter	Goiter	Goiter	Goiter	NA	
Age at reevaluation (year)	7	2	2	3	9	
Serum TSH at reevaluation (mU/L)	5.0	5.3	6.5	11.0	2.0	<5.0
Serum FT$_4$ at reevaluation (ng/dL)	1.7	1.4	NA	1.2	1.1	0.9-1.8
^{123}I uptake at 2 hours (%)	10.5	NA	NA	NA	NA	2-12
KClO$_4$ discharge rate (%)	18	NA	NA	NA	NA	<10

Abbreviations: NA, not available; NS, newborn screening. † Deleted genomic region included *DUOX2* and *DUOXA1* as well as *DUOXA2*.

DUOXA2 defect. There are two DUOXs and also two DUOXAs in the thyroid gland. Among four possible combination of the DUOX/DUOXA complexes, the DUOX1/DUOXA1 and DUOX2/DUOXA2 complexes have high H$_2$O$_2$-generating activity *in vitro* [18]. Considering the lower RNA expression levels of DUOX1 and DUOXA1 than DUOX2 and DUOXA2 [19], the DUOX1/DUOXA1 complex would function as a "backup" of the H$_2$O$_2$-generating system. If the *DUOX2* defect and the *DUOXA2* defect are regarded as the "DUOX2/DUOXA2 complex defect", one can reasonably expect that similar clinical phenotypes of the two genetic defects would arise.

Also notable is the elder brother of the proband, who did not have any histories suggesting hypothyroidism. He would have had normal or near-normal thyroid function in his newborn period, because the result of newborn screening for CH was negative. His first formal evaluation of thyroid function was conducted at age 9 years, and showed an undoubtedly normal serum TSH level. However, obviously high serum Tg level and marginally enlarged thyroid suggest that he would have a subtle defect in the hormone-producing capacity, which could be compensated by increasing the amount of thyroid follicular cells. At present, we could not define the factor(s) responsible for the phenotypic difference between the siblings. Generally, the thyroid-hormone producing capacity of newborns is easily influenced by environmental factors, such as transplacentally transferred iodine. Unrecognized genetic and/or environmental factor(s) might possibly underlie the phenotypic difference between the siblings. Similar phenotypic discordance between siblings with same genotype have been reported among *DUOX2* mutation carriers [20]. Thus, we presume that variable expressivity would be a common feature of the "DUOX2/DUOXA2 complex" defect.

In conclusion, we reported the fifth *DUOXA2* mutation-carrying CH patient. The patient showed age-dependent improvement of the thyroid hormone-producing capacity that was comparable to those of previous reports. We found that an apparently unaffected brother had the identical homozygous *DUOXA2* mutation. The observation implies that the phenotypic spectrum of *DUOXA2* mutations could be broader than currently accepted.

Acknowledgements

Disclosure

The authors have nothing to disclose.

Author Contributions

S.H., M.T. and Y.H. clinically characterized the study subjects and collected biological samples. C.S. and K.A. performed sequence analysis. C.S. conducted literature review. C.S. wrote the manuscript with critical input from S.N., M.T. and T.H.

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