Transient Congenital Hypothyroidism Caused by Biallelic Mutations of the Dual Oxidase 2 Gene in Japanese Patients Detected by a Neonatal Screening Program

Yoshihiro Maruo, Hiroko Takahashi, Ikumi Soeda, Noriko Nishikura, Katsuyuki Matsui, Yoriko Ota, Yu Mimura, Asami Mori, Hiroshi Sato, and Yoshihiro Takeuchi

Department of Pediatrics (Y.M., H.T., I.S., N.N., K.M., Y.O., Y.M., A.M., Y.T.) and Department of Lifescience (H.S.), Shiga University of Medical Science, Tsukinowa, Seta, Otsu, Shiga 520-2192, Japan

Context: Mutations in dual oxidase (DUOX2) have been proposed as a cause of congenital hypothyroidism. Previous reports suggest that biallelic mutations of DUOX2 cause permanent congenital hypothyroidism and that monoallelic mutations cause transient congenital hypothyroidism.

Objective: To clarify the inheritance of hypothyroidism, we looked at the DUOX2 gene in patients with transient congenital hypothyroidism.

Design: DUOX2, thyroid peroxidase, Na\(^{+}\)/I\(^{-}\) symporter and dual oxidase maturation factor 2 genes were analyzed in eight patients with transient congenital hypothyroidism, using the PCR-amplified direct sequencing method.

Patients: The eight patients were found by a neonatal screening program. Six of these patients belonged to two independent families; the other two were unrelated. Their serum TSH values varied from 24.8–233.0 mU/liter. Six of the eight patients had a low serum freeT\(_4\) level (0.19–0.84 ng/dl). Seven of the eight patients were treated with thyroid hormone replacement therapy, which ceased to be necessary by 9 yr of age.

Results: Eight novel mutations were detected in the DUOX2 gene. Four patients in one family were compound heterozygous for p.L479SfsX2 and p.K628RfsX10. Two patients in a second family were compound heterozygous for p.K530X and p.E876K;L1067S. The two remaining unrelated patients were also compound heterozygous, for p.H678R/p.L1067S and p.A649E/p.R885Q, respectively.

Conclusion: All eight patients had biallelic mutations in the DUOX2 gene. We find that loss of DUOX2 activity results in transient congenital hypothyroidism and that transient congenital hypothyroidism caused by DUOX2 mutations is inherited as an autosomal recessive trait. (J Clin Endocrinol Metab 93: 4261–4267, 2008)

Congenital hypothyroidism is one of the most common endocrine diseases in infants. The incidence of congenital hypothyroidism in neonatal screening programs is one in 4000 worldwide (1, 2). Early diagnosis of congenital hypothyroidism by such programs, and early replacement of thyroid hormone, prevents delayed motor and mental development (3). Thyroid hormone treatment for patients usually has to be lifelong (4). The etiology of congenital hypothyroidism is heterogeneous. The most common cause of congenital hypothyroidism is thyroid dysgenesis (aplasia, hypoplasia, or ectopic thyroid), which accounts for 75–80% of cases (5). Defects in T\(_4\) synthesis account for 15–20% of cases (5). Molecular studies have found that dysmorphogenesis in congenital hypothyroidism is caused by defects in genes involved in the synthesis of the following thyroid horm
miones: thyroid peroxidase (TPO), thyroglobulin (TG), sodium iodide symporter (NIS), and pendrin (PDS) (6–9). Many authors have proposed that inactivation of these enzymes causes permanent congenital hypothyroidism. Infants affected experience thyroid gland dysgenesis and defects in thyroid hormone-synthesizing enzymes, necessitating lifelong thyroid hormone replacement. The abnormality was transient in approximately 10–20% or more of neonates found in neonatal screening to be affected (this is transient congenital hypothyroidism: transient CH) (4, 10). Transient hypothyroidism can be caused by exposure of pregnant women having thyroid autoimmune disease to antithyroid medications, maternal antithyroid antibodies, iodine deficiency, or exposure to excess iodine in the perinatal period (10–13). Nose et al. (14) reported a genetic cause of transient CH, which is related to mild thyroid dyshormonogenesis. Infants with transient CH have to undergo replacement of thyroid hormone until their thyroid hormone production improves, to prevent psychomotor developmental delay (15).

The thyroid oxidase 2 (THOX) gene, known as dual oxidase 2 (DUOX2), has now been identified (16, 17). DUOX2 is located on chromosome 15 and consists of 33 exons encoding a mRNA 6376 nucleotides long. The DUOX2 protein is a 1548-amino-acid polypeptide, including a 26-amino-acid signal peptide. DUOX2 is located at the apical membrane of thyrocytes and is involved in the Ca2+/reduced nicotinamide adenine dinucleotide phosphate-dependent generation of H2O2. In the synthesis of thyroid hormone, TPO requires H2O2 to catalyze both the iodination of tyrosine residues and the coupling of iodotyrosine residues of TG (18). Previous reports have found that a defect in the system that generates H2O2 causes congenital hypothyroidism (19–21). Because defects in DUOX2 result in lack of H2O2, this protein is essential for thyroid hormone synthesis.

Moreno et al. (22) found by a screening program that four patients with congenital hypothyroidism (one permanent, three with transient hypothyroidism) had mutations of DUOX2. One patient with permanent congenital hypothyroidism (permanent CH), who was totally unable to organify iodine, was homozygous for a nonsense mutation (c.1300C→T, p.R434X) of DUOX2. The other three patients, having transient CH, were heterozygous for this mutation (p.Q686X, p.R701X, and p.S965fsX29, respectively) (22). In subsequent studies, nine additional mutations of DUOX2 were detected in patients with congenital hypothyroidism (p.Q36H, p.G418fsX62X, g.IVS19-2A→C, p.R842X, R376W, p.fsX300, p.D506N, p.Q1026X, and p.R1110Q) (23–27). Recently, another novel genetic cause of permanent CH has been found, namely dual oxidase maturation factor 2 (DUOX2A) (28). A genetic defect in DUOX2A impairs expression of DUOX2, resulting in decreased H2O2 production by thyrocytes.

Familial cases of transient CH are occasionally encountered in the neonatal screening program. Below, we analyze the DUOX2, TPO, NIS, and DUOX2A genes in six cases of familial transient CH in two families and two sporadic cases. We detected eight novel mutations of DUOX2. Previous studies assumed that heterozygous DUOX2 mutation causes transient CH, but the present eight cases of transient CH all had biallelic mutations of the DUOX2 gene.

Patients and Methods

Patients

We studied six patients in two unrelated families (four patients in family 1 and two patients in family 2) and two further unrelated patients. All eight patients were discovered from their elevated TSH levels in a neonatal screening program and consequently visited our hospital. In the course of clinical treatment and follow-up, infants showing improved thyroid function after withdrawal of replacement therapy were selected for the present study (Table 1). None had any history of deafness. The project was approved by the ethics committee of Shiga University of Medical Science.

Clinical report

Cases 1–4 (family 1)

The patients are the first, second, third, and fifth children of a nonconsanguineous couple. They were referred because of high serum TSH values detected in a neonatal screening program, at age 6–9 d. Cases 1–3 were female, and case 4 was male. At their first visit (age 15–28 d), the serum TSH values of cases 1–3 were between 95.4 to 233.0 mU/liter, and their serum free T4 (fT4) value was low at 0.19–0.53 ng/dl (see Table 1). Ultrasonography showed mild enlargement of the thyroid gland. After the diagnosis of hypothyroidism, cases 1–3 were treated by l-T4 supplementation. At age 7–9 yr, it was possible to cease the supplementation. Case 4 exhibited only moderate elevation of his serum TSH value (25.7 mU/liter). With no supplementation, his elevated TSH value had improved by 2 months of life. In recent clinical data for these four patients (aged 11, 7, 3, and 2 yr), their thyroid function parameters were within normal ranges, with no supplementation (Table 1). Motor and mental development of the patients was normal. The fourth brother had a normal TSH value (3.6 mU/liter) at neonatal screening (d 5 of life) and did not visit our hospital during his neonatal period.

Cases 5 and 6 (family 2)

These patients are sisters born to a nonconsanguineous couple. They were referred because of high serum TSH values found in the neonatal screening program at age 14 and 9 d. At their first visit (age 19 and 23 d), their serum TSH values were between 41.6 and 18.9 mU/liter and their fT4 values between 0.84 and 1.33 ng/dl (Table 1). Ultrasonography showed that their thyroid glands were normal in size. After the diagnosis of hypothyroidism, both patients underwent l-T4 supplementation. At age 2, l-T4 supplementation was discontinued for them both. In recent clinical data (ages 5 and 2 yr), their thyroid function parameters were within normal ranges in the absence of supplementation. Motor and mental development was normal.

Case 7

This patient is the first child of a nonconsanguineous couple; her serum TSH value in the neonatal screening program was 24.4 mU/liter. At her first hospital visit, serum TSH and fT4 were, respectively, 89.5 and 0.55 ng/dl (Table 1). Ultrasonography showed that her thyroid gland was normal in size. She received l-T4 supplementation. At age 4 yr, the supplementation was discontinued. Recent thyroid hormone values (age 4 yr) were within the normal range.

Case 8

This patient is the first child of a nonconsanguineous couple. Her serum TSH value in the neonatal screening program was 29.4 mU/liter. At her first hospital visit, serum TSH and fT4 values were, respectively, 24.8 and 0.62 ng/dl (Table 1). Ultrasonography revealed mild swelling of the thyroid gland. She underwent l-T4 supplementation until age 2 yr. Her recent thyroid hormone values (still aged 2 yr) were within the normal range.
Laboratory testing and sequence analysis

At neonatal screening, TSH was measured by HPLC. TSH was measured at hospital visits using a fluorescent enzyme immunoassay method (Tosho, Tokyo, Japan). fT4 and fT3 were measured by fluorescent enzyme immunoassay method at hospital visits using a fluorescent enzyme immunoassay method. 

TG was measured by an immunoradiometric assay (Eiken Chemical, Tokyo, Japan). 

Genomic DNA was extracted from blood leukocytes using standard techniques, after individuals and their parents had given informed consent to participate in this study. Amplification of exons and the exon-intron boundaries by PCR from genomic DNA were performed using the 10 pairs of oligonucleotide primers specified in the supplemental table (published online at the Endocrine Society’s Journals Online web site at http://jcem.endojournals.org). The genomic DNA sequence and cDNA sequences were based on the human genome database (NCBI accession numbers CCDS10117.1 and AP267981). The 33 exons of DUOX2 were amplified into 10 PCR products. PCR products were purified using SUPREC 02 (TaKaRa, Kyoto, Japan), and the sequences of the amplified DNA fragments were determined directly using BigDye Terminators version 1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The sequencing primers are shown in the supplemental table.

The PCR products, including novel mutations of DUOX2, were subcloned to pCR 2.1 vectors using a TA-cloning kit (Invitrogen, San Diego, CA), to determine changes in the nucleotide sequence. We ligated 30 ng PCR fragments, including the mutation regions, into the 50-ng vector; transformation by the products was performed using a Competent High JM109 kit (Toyobo, Osaka, Japan). The relatives of the patients, and also 50 healthy Japanese volunteers, had their DUOX2 analyzed for genotyping and detection of polymorphisms, with informed consent in all cases.

For analysis of the genes encoding TPO and NIS, all coding exons and surrounding intronic sequences were amplified by PCR and were sequenced directly as described previously (29, 30). For analysis of the genes encoding DUOX2A, the coding exons and the surrounding intronic sequences were amplified by PCR using the primers 5’-TACCAAGCCCAGGCGTTGAG3’ and 5’-GGCAGGCGTTTCTGCAAGT-3’. An amplified DNA fragment was sequenced directly using the primer 5’-CAGGACGCCAGCTTGTGCTG-3’ for exon 1 and 5’-CTAGGTGTTGCACTTTCCAG-3’ for exon 2.
Results

Identification of mutations by sequencing of DUOX2 in patients with transient CH

Family 1: cases 1–4

Direct sequencing of genomic DNA revealed that all four patients had bilallelic mutations of DUOX2. One is a deletion-insertion mutation in exon 12 of the DUOX2. The nucleotide sequence of CTATCC at 1435_1440 was replaced by AG. This deletion-insertion leads to a frameshift producing a stop at codon 481: c.1435_1440delCTATCCinsAG (p.L479SfsX2) (Fig. 1). Another mutation is deletion of an adenine at position 1883 in exon 15 (Fig. 1). This frameshift mutation generates a stop codon at 638: c.1883delA (p.K628RfsX10). The unaffected father and brother were heterozygous for c.1435_1440delCTATCCinsAG, and the mother was heterozygous for c.1883delA (Fig. 2A).

Family 2: cases 5 and 6

Direct sequencing of genomic DNA revealed that both patients have three novel mutations. One is a nonsense mutation: a transition A to T at 1588 in exon 13, which changes lysine at codon 530 to a stop codon: c.1588A>T (p.K530X) (Fig. 1). The second mutation is a missense mutation by a transversion of G to A at 2635 in exon 19, which replaces glutamic acid with lysine at codon 879: c.2635G>A (p.E879K) (Fig. 1). The third mutation is a missense mutation by a transversion of T to C in exon 24, which replaces leucine with serine at codon 1067: c.3200T>C (p.L1067S) (Fig. 1). The patients were compound heterozygous for the double point mutations, c.1588A>T;3200T>C (Fig. 2B).

Case 7

This patient was compound heterozygous for two missense mutations. One is a transversion mutation A to G, which replaces histidine with arginine: c.2033A>G (p.H678R) in exon 16 (Fig. 1). The other is c.3200T>C (p.L1067S) in exon 24 (Fig. 1). The mother was heterozygous for c.2033A>G, and the father was heterozygous for c.3200T>C (Fig. 2C).

Case 8

The patient was compound heterozygous for two missense mutations. One is a transition mutation C to A at nucleotide 1946, which replaces alanine with glutamic acid at codon 649 in exon 16; c.1946C>A (p.A649E) (Fig. 1). The other is a transversion mutation of G to A at nucleotide 2654, which replaces arginine with glutamine at codon 885 in exon 19: c.2654G>A (p.R885Q) (Fig. 1). The mother was heterozygous for c.2654G>A, and the father was heterozygous for c.1946C>A (Fig. 2D).

The seven DUOX2 mutations described above were absent in 100 alleles from Japanese healthy volunteers, indicating that the mutations are not polymorphisms. Only one allele had c.3200T>C. The allelic frequency of the mutation is 0.005,
indicating that c.3200T→C is not a polymorphism in the Japanese population. This mutation could nevertheless be a rare polymorphism in the Japanese population, because it was detected in two double-mutation alleles in cases 5 and 6.

None of the patients had any additional mutations of DUOX2, NIS, or TPO, except for previously reported TPO polymorphisms (31). We have submitted the sequence data for the DUOX2 mutations to the DDBJ/EMBL/GenBank databases under the accession numbers AB379823, AB379824, AB379825, AB379826, AB379827, AB379828, AB379829, and AB379830.

**Discussion**

The causes of transient CH have been assumed to be excessive intake or, alternatively, lack of iodine, or transplacental antibody or antithyroid drugs from the mother (10–13). Although the diet of pregnant women has been scrutinized to promote intake of iodine in the appropriate range, transient CH onset has not disappeared. Transplacental antibody migration is an important cause of transient CH (10–15). Before DNA analysis, we expected that the present eight cases, especially the familial cases, would be heterozygous for mutations in the DUOX2 gene. Surprisingly, all patients had biallelic mutations in the DUOX2 gene. Four cases (cases 1–4 in family 1) were compound heterozygous for the frameshift mutations (p.L4795fsX2 and p.K628RfsX10), which suggests total loss of DUOX2 activity. The serum TSH value of the unaffected fourth brother in the family, who was heterozygous for p.L4795fsX2, was 3.6 mU/liter; he did not display transient CH. Moreover, the fifth brother (case 4), with the compound heterozygous frameshift mutations, exhibited only transient elevation of TSH values (hyperthyrotropinemia); his TSH value improved within 3 months of birth to less than 5 mU/liter without replacement therapy. In family 2 (cases 5 and 6), both sisters were compound heterozygous for a nonsense mutation p.K530X and a double missense mutation p.E879K;L1067W; they also exhibited transient CH. The sporadic cases 7 and 8 were also compound heterozygous for missense mutations: p.H678R/p.L1067S and p.A649E/p.R885Q, respectively. According to analysis of 100 alleles of DUOX2 derived from healthy Japanese volunteers, the eight novel DUOX2 mutations detected in this study are not polymorphisms in the Japanese population. The present study demonstrates, in contrast to previous reports, that biallelic mutations of the DUOX2 gene cause transient CH. The results in family 1 clearly indicate that complete inactivation of DUOX2 does not cause permanent CH, but rather causes transient CH.

We believe that complete inactivation of DUOX2 causes transient CH but not permanent CH for the following reasons. 1) An additional H2O2-generating oxidase exists in thyrocytes, namely DUOX1 (16, 17), although the exact role of DUOX1 in thyroid hormone synthesis has not been determined. The amount of DUOX1 expressed is one fifth that of DUOX2 (33). Even if enzyme activity of DUOX2 is lost, a low level of H2O2 supply caused by DUOX1 expressed is insufficient for the synthesis of thyroid hormone (16, 17). Thirteen mutations of DUOX2 have been reported, and it has been proposed that monoallelic mutations of the DUOX2 gene cause transient CH (22–27). No familial transient congenital hypothyroidism with dominant trait caused by the DUOX2 gene has been reported, however. Familial cases of transient CH exist, and a genetic cause for transient hypothyroidism has been suggested, namely heterozygous mutations of DUOX2 (22). In most cases, the causes of transient CH were unclear (14, 32).

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times that of an adult (2 μg/kg) (34, 35); thyroid hormone requirement gradually decreases after the infantile period. The H₂O₂ supply from DUOX1 alone may be inadequate in the neonatal and infantile period, so that carriers with DUOX2 deficiency are liable to show transient congenital hypothyroidism.

Vigone et al. (23) found patients with biallelic mutations of DUOX2 who have a mild phenotype of congenital hypothyroidism. Case 4, with the same compound heterozygous frameshift mutations as cases 1–3, exhibited only transient elevation of TSH (transient hyperthyrotropinemia); it is possible that other genetic factors, such as DUOX2, modify the H₂O₂ supply by DUOXs (26). Moreover, previous reports of DUOX2 mutations suggest that monoaletic mutations cause transient congenital hypothyroidism (22–25). Discrepancies in these findings could be due to ethnic differences in the genes involved in thyroid hormogenesis. Ohye et al. (26) recently reported a case of adult-onset hypothyroidism due to iodine organization defect with homozygous DUOX2 mutation (p.R1110Q). The patient was initially euthyroid but later developed hypothyroidism in her 40s. Our present study and the case with p.R1110Q indicate that biallelic mutations of DUOX2 cause transient CH in the neonatal period and lead later to the development of adult-onset hypothyroidism with senescence.

Transient CH caused by external factors should take a transient course. However, in patients with transient CH caused by dysmorphogenesis based on genetic defects, such as loss of DUOX2 activity, subclinical reduction of hormone synthesis may continue throughout life. After improvement in the serum hormone level, there is still a risk of hypothyroidism recurring when there is an increased requirement for thyroid hormone (36). An example is during pregnancy; this is known as maternal hypothyroditism and affects the neuropsychological development of the fetus (37). Identification of genetic causes of transient CH in patients with no obvious external symptoms of hormone defect is therefore important in maintaining their subsequent health.

In this study, we detected eight novel mutations in DUOX2 in Japanese patients with transient CH. In each case, the patient had a biallelic mutation in the DUOX2 gene. This indicates that loss of DUOX2 activity results in transient CH, and there are both autosomal dominant and recessive transient types of CH (22). In the cases in family 1, for instance, identical mutations did not give rise to the same phenotype, implying the existence of additional factors that influence the clinical and biochemical expression of congenital hypothyroidism. Further studies, including family members and descendants of patients, are necessary to settle the factors causing transient CH in relation to DUOX2.

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Address all correspondence and requests for reprints to: Yoshihiro Maruo, M.D., Ph.D., Department of Pediatrics, Shiga University of Medical Science, Tsukinowa, Seta, Otsu, Shiga 520-2192, Japan. E-mail: maruo@belle.shiga-med.ac.jp.

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