Identification and Functional Studies of Two New Dual-Oxidase 2 (DUOX2) Mutations in a Child with Congenital Hypothyroidism and a Eutopic Normal-Size Thyroid Gland

Massimo Tonacchera, Giuseppina De Marco, Patrizia Agretti, Lucia Montanelli, Caterina Di Cosmo, Andrea Claudia Freitas Ferreira, Antonio Dimida, Eleonora Ferrarini, Helton Estrela Ramos, Claudia Ceccarelli, Federica Brozzi, Aldo Pinchera, and Paolo Vitti

Dipartimento di Endocrinologia e Metabolismo (M.T., G.D.M., P.A., L.M., C.D.C., A.D., E.F., C.C., F.B., A.P., P.V.), Centro Eccellenza AmbiSEN, Università di Pisa, 56124 Pisa, Italy; Laboratorio de Fisiologia Endocrina (A.C.F.F.), Instituto de Biofisica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, 21941-901 Brazil; and Division of Endocrinology and Metabolism (H.E.R.), Department of Medicine, Federal University of Paraná, Curitiba, 80030-110 Brazil

Context: Some cases of congenital hypothyroidism (CH) are associated with a gland of normal size.

Objective: To explore the cause of organification defect in one child with CH and a eutopic thyroid gland, genetic analyses of TPO, DUOX2, and DUOXA2 genes were performed.

Patient: One child with CH, a eutopic thyroid gland, and a partial organification defect was shown after ^{123}I scintigraphy and perchlorate test.

Methods: In the child with the organification defect, TPO, DUOX2, and DUOXA2 genes were analyzed. The functional activity of the DUOX2 mutants was studied after expression in eukaryotic cells.

Results: No TPO or DUOXA2 gene mutations were identified. Direct sequencing of the DUOX2 gene revealed a compound heterozygous genotype for S911L and C1052Y substitutions. S911L and C1052Y caused a partial defect in H_2O_2 production after transient expression in HeLa cells.

Conclusions: We performed a genetic analysis in one child with CH and a eutopic thyroid gland. Two new mutations in DUOX2 gene responsible for the partial deficit in the organification process were identified. **(*J Clin Endocrinol Metab* 94: 4309–4314, 2009)**

Congenital hypothyroidism (CH) is most commonly due to defects in thyroid development leading to glandular dysgenesis. In about 15% of cases, CH is associated with the presence of goiter or with a eutopic gland of normal size (1). Molecular studies have found that dyshormogenesis in CH is caused by defects in genes involved in the synthesis of thyroid hormones such as thyroid peroxidase (TPO), thyroglobulin (TG), sodium iodide symporter (NIS), and pendrin (PDS).

The thyroid oxidase 2 (THOX) gene, known as dual oxidase 2 (DUOX2, accession no. NM_014080) has now been identified (2, 3). DUOX2 is located on chromosome 15 and consists of 33 exons. 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 Ca^{2+}-reduced nicotinamide adenine dinucleotide phosphate-dependent generation of H_2O_2. Recently, a novel gene has been cloned,

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Abbreviations: CH, Congenital hypothyroidism; DUOX2, dual oxidase 2; FT_4, free T_4; HA, hemagglutinin; TG, thyroglobulin; TPO, thyroid peroxidase; wt, wild type.

the product of which (dual oxidase maturation factor 2; DUOXA2, accession no. NM_207581) is required to express DUOX2 enzymatic activity (4). The generation of H_2O_2 is a critical step in thyroid hormonogenesis (5). The essential role of DUOX2 in thyroid hormone synthesis has been confirmed by the recent observation that monoallelic inactivating mutations of DUOX2 gene are present in children with transient CH (6). In this paper, we investigated the etiology of CH in one child identified at neonatal screening with a normal eutopic thyroid gland. When the child was 14 yr old, a thyroid scintiscan was performed, and an organification defect was shown after perchlorate administration. In this child, TPO and DUOX2 and DUOXA2 genes were studied. Two new mutations in the DUOX2 gene were identified.

Case reports

The patient is a female detected by the neonatal screening program for CH. She was born at term by cesarean section after an uncomplicated pregnancy from Italian unrelated healthy parents. Her birth weight was 3680 g, length 49 cm, and cranial circumference 35 cm. Her neonatal history was characterized by a 10-d-long jaundice. Spot blood TSH was 21. The diagnosis of CH was confirmed by serum thyroid function tests (TSH 32 mU/liter, normal values 0.4–8; free T_4 (FT_4) 6.9 pg/ml, normal values 7–18; FT_3 4.2 pg/ml, normal values 2.5–5.5; TG 40 ng/ml, normal values <1–50), and L-T_4 replacement therapy was started immediately, after the first evaluation, at the age of 20 d at an initial dose of 6 μg/kg · d. Data from the maternal medical history excluded thyroid diseases, hearing problems, or the use of medications affecting thyroid function.

Replacement therapy was modified during follow-up according to clinical and hormonal evaluation to maintain normal serum TSH and serum FT_4 in the upper normal range. At 5 yr of age, after a temporary withdrawal of L-T_4 therapy for 6 wk, an ^{123}I thyroid scan showed a thyroid gland of normal size in the proper position in the neck. Thyroid function evaluation confirmed a subclinical hypothyroidism (FT_4 10.4 pg/ml, normal values 7–18; FT_3 3.5 pg/ml, normal values 2.5–5.5; TSH 6 mU/liter, normal values 0.4–3.6). Physical and mental development proceeded normally. Heart was normal by two-dimensional echocardiography. Bone age corresponded to the chronological age. Ultrasonography of the abdomen was normal. On x-ray, no abnormalities at hips were observed.

Menstrual cycles were regular because menarche at age of 14 yr and final height was appropriate to her genetic target (158 cm), and weight was 73 kg. Moreover, she completed a high school education.

At 14 yr old thyroid echography confirmed the presence of a normal-sized thyroid gland (volume 15 ml) with a normoechogenic pattern, and a perchlorate discharge test was performed.

At 3 h after administration of ^{123}I, 2.96 MBq, an anterior image of the thyroid was acquired for 5 min with a γ-camera equipped with a low-energy high-resolution collimator. ^{123}I uptake was determined using a region of interest technique, comparing the counts over the thyroid with the counts of a standard. At 3 h, potassium perchlorate ($KClO_4$) was administered orally (400 mg), and 1 h later, another acquisition was repeated with the same method.

The controls were 20 adult subjects with no thyroid disorders (perchlorate discharge test, 0.5–2.5%).

One month after L-T_4 suspension, FT_4 was 6.8 pg/ml (normal values 7–17), FT_3 was 2.8 pg/ml (normal values 2.5–5.5), TSH 8.2 mU/liter (normal values 0.4–3.6), and TG 30 ng/ml (normal values <1–50). Serum anti-TG and anti-TPO antibodies were not measurable. Urinary iodide was 50 μg/liter. The perchlorate test showed a slight organification defect (13%). L-T_4 therapy was started again, and she remained euthyroid with a low dose.

Family members

Her parents were clinically euthyroid, and their serum tests of thyroid function were in the normal range; anti-TPO and -TG antibodies were not detectable. Both parents had a normal-sized thyroid gland on ultrasound.

The parents gave informed consent to undergo studies approved by the local ethical committee.

Controls

Sixty healthy subjects not affected by thyroid disorders were included in the study (mean age 39 yr, range 11–66).

Materials and Methods

Laboratory evaluation of thyroid function

Serum FT_4 and FT_3 were measured with a RIA (FT_4 RIA, FT_3 RIA; Lysophase, Technogenetics SpA, Milan, Italy). TSH was assessed by sensitive TSH immunoradiometric assay (Delfia, Wallac, Finland). TPO and TG antibodies were measured by passive agglutination (SERODIA-AMC and SERODIA-ATG; Fujirebio, Tokyo, Japan).

Sequence determination

Genomic DNA was extracted from peripheral lymphocytes using standard procedures (6). All 17 coding exons for TPO were PCR amplified and sequenced as described previously (7). All 33 coding exons for DUOX2 were PCR amplified and sequenced as described in Moreno *et al.* (6). DUOXA2 was sequenced as described previously (4).

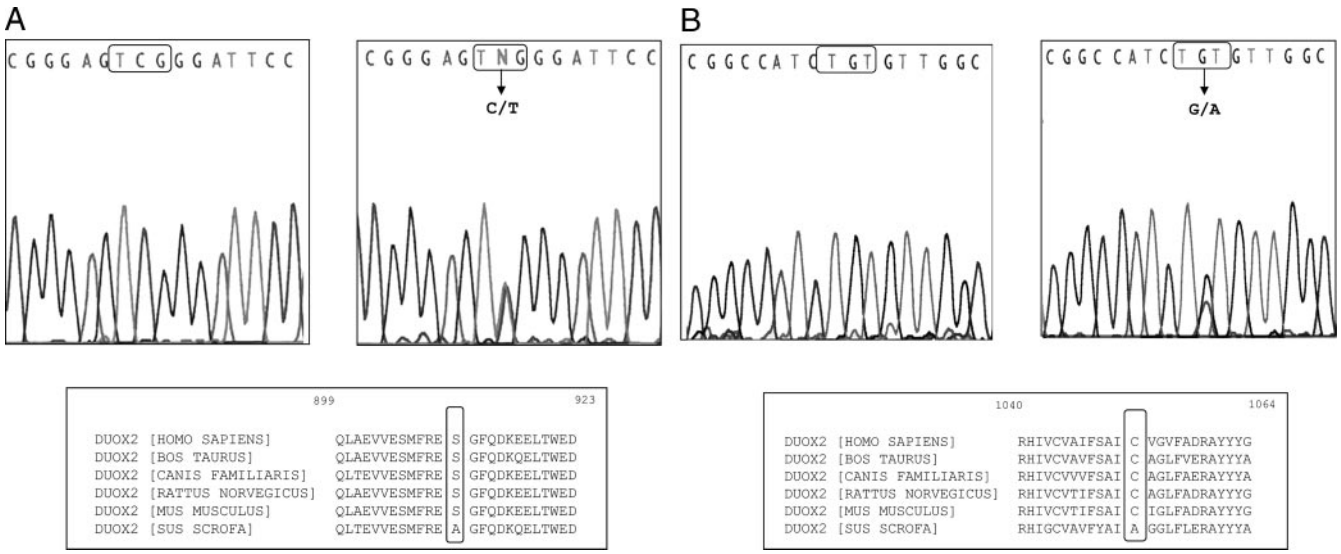


FIG. 1. Automated fluorescence-based sequencing chromatograms of the DUOX2 gene showing the mutants S911L (A) and C1052Y (B). In *boxes*, the degree of conservation of the different modified residue in mammalian DUOX2 proteins is shown.

Construction of the expression vector and functional analysis

Preparation of wild-type (wt) DUOX2 and DUOXA2 in the expression vector pcDNA3 were as described (8). Mutants harboring the single-nucleotide missense substitutions were generated by site-directed mutagenesis using the Gene Tailor site-directed mutagenesis system (Invitrogen Life Technologies, Carlsbad, CA). The accuracy of the recombinant constructs was verified by direct sequencing.

HeLa cells were maintained in high-glucose DMEM supplemented with 50 U/ml penicillin, 50 mg/ml streptomycin, and 10% fetal bovine serum under humidified 5% CO_2 at 37 C (Invitrogen). For functional studies, cells were seeded in 12-well plates at the concentration of about 100,000 per well and after 24 h were cotransfected with 250 ng wt DUOX2 and DUOX2 mutants or with the empty vector in the presence of 50 ng DUOXA2 by using Fugene 6 transfection reagent and following the manufacturer's instructions (Roche Applied Science, Indianapolis, IN). To simulate the compound heterozygous state of the proband, HeLa cells were also transfected with 125 ng of both mutations in the presence of DUOXA2. Green fluorescence protein expression from a cotransfected pEGFP vector (Clontech Laboratories, Palo Alto, CA) was used to monitor transfection efficiency in whole cells.

Forty-eight hours after transfection, cells were assayed for the generation of H_2O_2 as described (8). Release of H_2O_2 was determined by reaction with cell-impermeable 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red reagent; Invitrogen) in the presence of excess peroxidase, producing fluorescent resofurin. Briefly, cell monolayers were incubated with or without 10 μM of the H_2O_2 production inhibitor diphenyleneiodonium, in Dulbecco's PBS supplemented with 50 mM Amplex Red reagent and 0.1 U/ml horseradish peroxidase for 1 h at 37 C. Relative fluorescence units (excitation/emission 535–595 nm) of the medium were measured within the linear range of the H_2O_2 concentration response curve by using Picofluor fluorometer (Turner Designs, Sunnyvale, CA). Changes in fluorescence intensity were converted into absolute nanomoles of H_2O_2 using a calibration curve.

The expression of wt and mutated DUOX2 proteins was determined in HeLa cells 48 h after transfection by microchip flow cytometry analysis using an Agilent 2100 bioanalyzer (Agilent Technologies, Deutschland GmbH, Waldbronn, Germany) as previously described (9, 10). The primary anti-hemagglutinin (anti-HA) antibody 3F10 (Roche Applied Science, Indianapolis, IN) was used to detect HA-conjugated DUOX2 proteins at the concentration 200 ng/μl. Affinity-purified Alexa Fluor 647 goat antirat IgG (Molecular Probes, Invitrogen) was used as secondary antibody. Results are expressed as percentage of antibody-stained cells within the live population.

Results

Direct sequencing of the TPO and DUOXA2 genes did not show any mutation (data not shown).

Direct sequencing of the 33 coding exons of DUOX2 gene revealed that the child was compound heterozygous for S911L and C1052Y substitutions (Figs. 1, A and B, and 2). The mother harbored the S911L and the father the C1052Y substitution; both of them were euthyroid. The S911 is located in the cytosolic domain with the EF-hand motifs, whereas the C1052 is located in the second transmembrane segments (Fig. 2). S911 and C1052 are amino acids highly conserved in other mammalian species (Fig. 1, A and B) and modifications of these residues have not been found in the screening of 120 alleles from healthy subjects.

Functional studies

The functional activity of new DUOX2 mutants was studied after expression in HeLa cells (Fig. 3). Cells were cotransfected with either wt or one or both of the mutant (S911L and C1052Y) DUOX2 cDNAs alone or together with equal amount of DUOXA2 expression vector. Forty-eight hours later, the amount of H_2O_2 generated was measured, and the level of DUOX2 surface expression was determined.

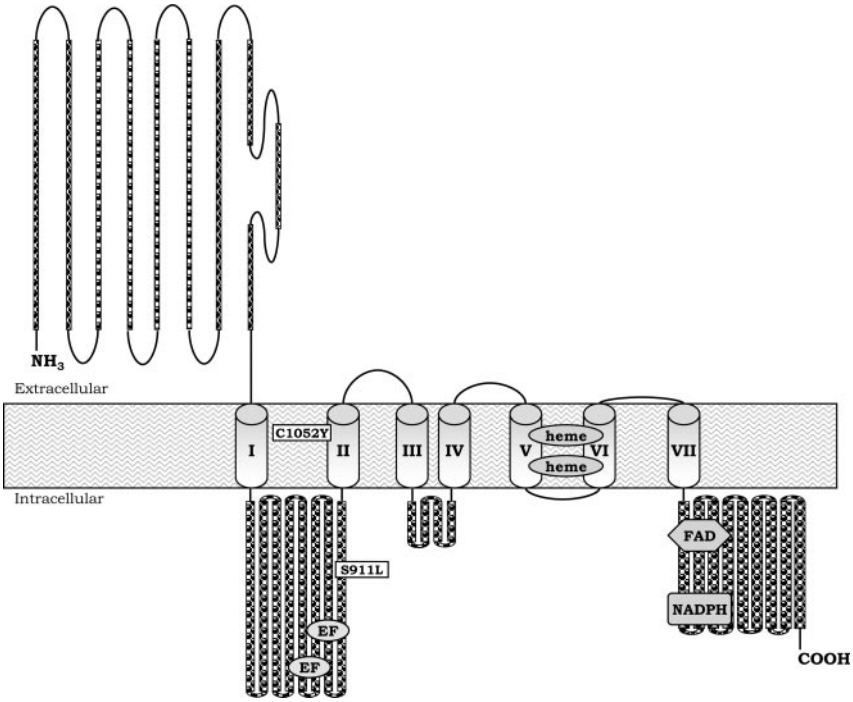


FIG. 2. A topological model of DUOX protein with the localization of the mutants identified in this manuscript.

The cotransfection of wt DUOX2 with DUOXA2 rescued DUOX2 activity as indicated by the significant amounts of H_2O_2 released from the cells. The S911L and C1052Y mutations significantly impaired the H_2O_2-generating system in the same experimental conditions, suggesting that these mutants were able to partially inhibit the functional activity of the H_2O_2-generating system (Fig. 3). The presence of both mutations also significantly reduced the H_2O_2 production in HeLa cells (Fig. 3).

The level of cell surface expression of DUOX2 proteins was measured by microchip flow cytometry analysis using the anti-HA monoclonal antibody 3F10 in the presence of DUOXA2 protein (Fig. 4).

The mutants S911L and C1052Y showed a significantly lower expression at the cell surface with respect to the wt DUOX2 (46.9 and 57.6%, respectively) when individually transfected in HeLa cells; cotransfection of cells with both mutants also determined a reduction of DUOX2 surface expression (56.3%).

Discussion

We describe a new case of CH with a normally located thyroid gland. At 14 yr of age, a thyroid function test after L-T_4 discontinuation showed a mild hypothyroidism, a thyroid of normal size in the proper position in the neck, an intact iodide trapping, and a partial organification deficit. Molecular analyses revealed that the affected child was compound heterozygous for two mutations (S911L and C1052Y) in the DUOX2 gene. No mutations in the TPO and DUOXA2 genes were found.

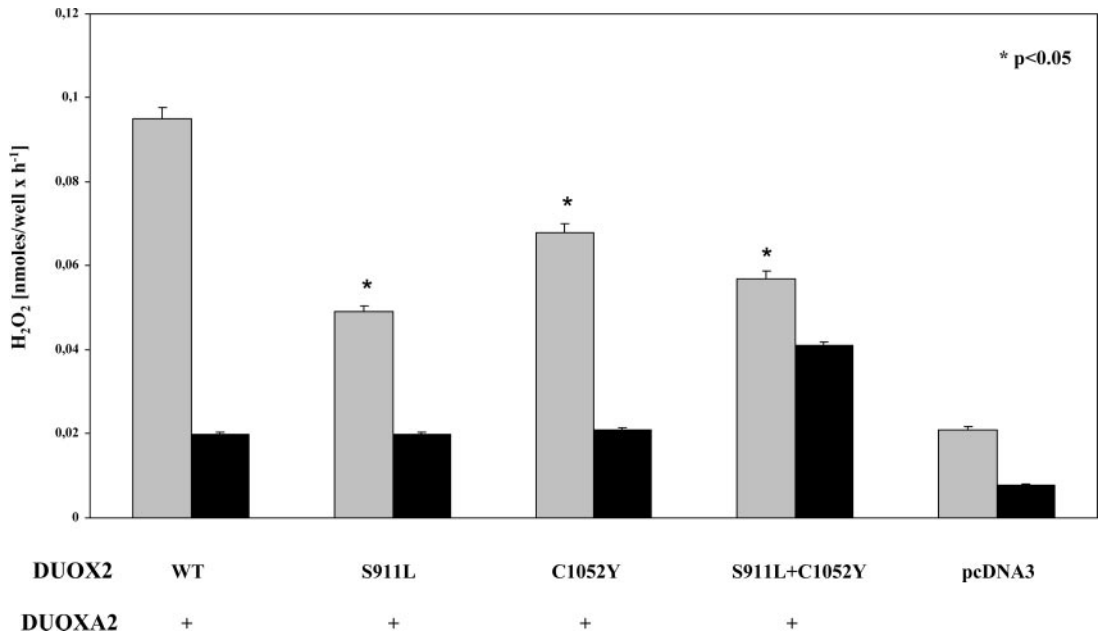
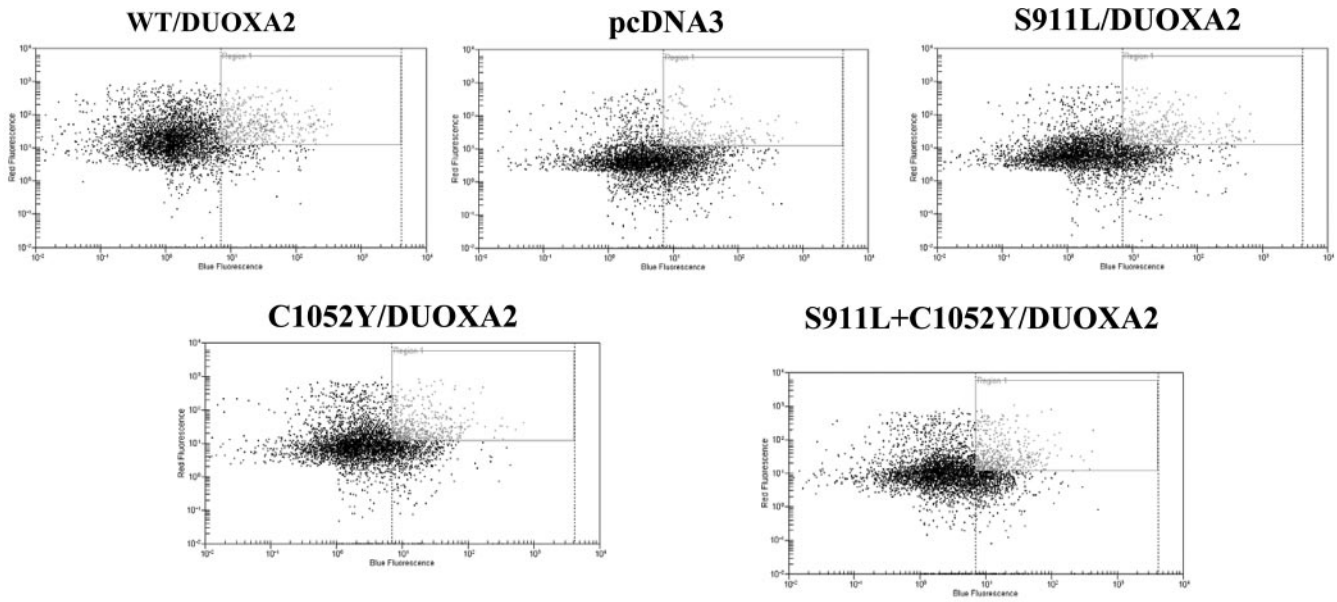


FIG. 3. DUOX2-mediated H_2O_2 generation in HeLa cells transfected with the indicated expression vectors. The total amount of DNA per transfection was kept constant by adjusting with empty vector. *Gray columns*, H_2O_2 production induced by cotransfection of DUOX2 with DUOXA2; *black columns*, the same experiments in the presence of diphenyleneiodonium, an inhibitor of H_2O_2 production; *, statistically different.



	WT/DUOXA2	pcDNA3	S911L/DUOXA2	C1052Y/DUOXA2	S911L+C1052Y/DUOXA2
% gated events	73.3	16.9	34.4	42.2	41.3
% of the WT	100	23.1	46.9	57.6	56.3

FIG. 4. Expression analysis of DUOX2 mutations obtained by microchip flow cytometry using an anti-HA antibody. Cells were stained with the live cell dye calcein and with the antibody. Results are expressed as percentage of antibody-stained cells within the live population (corresponding to percentage of gated events). The percentage of expression at the cell surface of mutants with respect to the wt DUOX2 was also reported.

Each parent was heterozygous for one of the two mutations, and thyroid function and morphology were completely normal. The S911 is located in the cytosolic domain with the EF-hand motifs, whereas the C1052 is located in the second transmembrane segments. S911 and C1052 are highly conserved in other mammalian species and have not been found in the screening of 120 alleles from healthy subjects. The functional study after coexpression in HeLa cells with DUOXA2 clearly showed partial loss of H_2O_2-generating activity, which provides a link with the iodine organification defect of this patient. The mutants S911L and C1052Y showed a lower expression at the cell surface with respect to the wt DUOX2 when individually transfected in HeLa cells; cotransfection of cells with both mutants also determined a reduction of DUOX2 surface expression, suggesting that the mutants are responsible for a partial defect of the routing of the DUOX2 with reduced targeting to the cell surface. These observations show that biallelic inactivating mutations of the DUOX2 may be responsible for cases of mild hypothyroidism associated with partial defect in the organification process.

Moreno *et al.* (6) described that biallelic inactivating mutations in the DUOX2 gene result in complete disruption of thyroid hormone synthesis and are associated with severe and permanent CH. Monoallelic mutations are associated with milder, transient hypothyroidism caused by insufficient thyroidal production of hydrogen peroxide, which prevents the synthesis of sufficient quantities of thyroid hormones to meet the large requirement of thyroid hormones at the beginning of life.

Two siblings with persistent mild hypothyroidism and a partial organification defect were also described (11). Both children were compound heterozygous for two DUOX2 variants, a nonsense mutation (Arg842X) and a missense substitution (Arg376Trp). Two other unknown compound heterozygous mutations in the DUOX2 gene, p.Q36H/p.S965fsX994 and p.G418fsX482/g.IVS19-2A→C have been identified (12) and are responsible for iodide organification defects in two unrelated families. Pfarr *et al.* (13) described an ins602-FsX300 heterozygous child with CH and a compound heterozygous for ins602/D506N. The D506N was shown to cause a partial defect when expressed *in vitro* (8). Whereas the six individuals with mutations in both alleles had persistent primary hypothyroidism, three patients with monoallelic mutations causing truncations had transient hypothyroidism. The last three individuals had

mild hypothyroidism during the newborn period and were treated with low doses of L-T_4. At 3 yr of age, after L-T_4 withdrawal, they remained euthyroid. One explanation of this phenomenon is that partial insufficiency of the DUOX protein may have an effect during early postnatal life, when more thyroid hormone is required. This is in agreement with the observation that most heterozygous individuals so far described have normal thyroid function when investigated later in life despite a partial iodide organification defect. Contrary to what has been described previously, very recently, Maruo *et al.* (14) reported eight children (six belonging to two families and two sporadic) with transient CH and normally located thyroid gland harboring biallelic mutations in the DUOX2 gene. The same authors speculated that the complete inactivation of DUOX2 caused transient and not permanent CH because an additional H_2O_2-generating system exists in thyrocytes, namely DUOX1. The H_2O_2 supply from DUOX1 alone may be inadequate in the neonatal and infantile period, so that carriers with DUOX2 deficiency are liable to show transient CH.

The previous clinical descriptions and our clinical seminar clearly show that biallelic impairment of the DUOX2 can be associated with severe but also a mild form of hypothyroidism. The reason for this discrepancy is not known, but the influence of ethnic differences in the genes involved in thyroid hormonogenesis or the different iodide supply in the populations are possible explanations. We must also consider that Ohye *et al.* (15) recently reported a case of adult-onset hypothyroidism due to iodine organification defect with homozygous DUOX2 mutations, but in this case, a functional study on the activity of the mutated protein is not demonstrated.

In conclusion, we describe a new case of CH with a normally located thyroid gland. Two new mutations in the DUOX2 protein have been found, and the functional study in a heterologous system confirmed the partial impairment of the H_2O_2 generation.

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Address all correspondence and requests for reprints to: Massimo Tonacchera, Dipartimento di Endocrinologia, Università di Pisa, Via Paradisa 2, 56124, Cisanello, Pisa, Italy. E-mail: mtonacchera@hotmail.com.

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