The Clinical and Molecular Characterization of Patients With Dyshormonogenic Congenital Hypothyroidism Reveals Specific Diagnostic Clues for DUOX2 Defects

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Context: Mutations in the *DUOX2* gene have been associated with transient or permanent congenital hypothyroidism due to a dyshormonogenic defect.

Objective: This study aimed to verify the prevalence of *DUOX2* mutations and the associated clinical features in children selected by criteria supporting a partial iodide organification defect (PIOD).

Patients and Methods: Thirty children with PIOD-like criteria were enrolled and genotyped. A detailed clinical characterization was undertaken together with the functional analysis of the *DUOX2* variations and the revision of the clinical and molecular data of the literature.

Results: In this large selected series, the prevalence of the *DUOX2* mutations was high (37%). We identified 12 missense variants, one splice site, and three frameshift *DUOX2* mutations. Functional analyses showed significant impairment of H_2O_2 generation with five missense variants. Stop-codon mutants were shown to totally abolish DUOX2 activity by nonsense-mediated RNA decay, exon skipping, or protein truncation. *DUOX2* mutations, either mono- or biallelic, were most frequently associated with permanent congenital hypothyroidism. Moreover, the present data suggested that, together with goiter and PIOD, the most significant features to select patients for the *DUOX2* analysis are the low free T_4 and the high TSH concentrations at the first postnatal serum sampling, despite borderline blood spot TSH. Interestingly, the analysis of previously described *DUOX2* mutated cases confirmed the validity of these findings.

Conclusions: The defects in the peroxide generation system are common among congenital hypothyroidism patients with PIOD. The most robust clinical parameters for selecting patients for DUOX2 analysis have been identified, and several *DUOX2* variants have been functionally characterized. **(*J Clin Endocrinol Metab* 99: E544–E553, 2014)**

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Abbreviations: bsTSH, blood spot TSH; CH, congenital hypothyroidism; fT4, free T_4; $KClO_4^-$, perchlorate; MIM, Mendelian Inheritance in Man; PIOD, partial iodide organification defect; sfT4, serum free T_4; SNP, single-nucleotide polymorphism; sTSH, serum TSH; Tg, thyroglobulin; TPO, thyroperoxidase; wt, wild type.

Congenital hypothyroidism (CH) is one of the common preventable causes of mental retardation, and neonatal screening programs allow the early detection and treatment of CH cases. Its incidence is variable among different countries and even within the same country, ranging from 1:2000 to 1:3500 newborns per year (1). In our Italian region, a high incidence of 1:1446 has been recorded, mainly due to the shift of screening TSH cutoffs down to 10 mU/L (2).

Although CH can be also caused by dysgenetic defects, most CH cases have recently been found to be more frequently associated with altered function of an otherwise normally positioned thyroid gland (2). In particular, dyshormonogenic defects include alterations in one of the players involved in biosynthesis of thyroid hormones, such as pendrin [*PDS* (Mendelian Inheritance in Man [MIM]*605646)], thyroperoxidase [*TPO* (MIM*606765)], and thyroglobulin [*Tg* (MIM*188450)]. A novel metabolic pathway involving the complex DUOX2/DUOXA and devoted to the generation of the hydrogen peroxide (H_2O_2) needed for TPO activity (3, 4), has been shown to play a crucial role in thyroid hormonogenesis. *DUOX2* [*DUOX2* (MIM*606759)] gene maps on chromosome 15 and encodes a protein comprising a N-terminal peroxidase-like domain, seven transmembrane domains, two cytosolic Ca^{2+}-binding sites (EF-hand motifs), and a C-terminal oxidase domain, including binding sites for nicotinamide adenine dinucleotide phosphate and flavin adenine dinucleotide. The maturation factors DUOXA1 [*DUOXA1* (MIM*612771)] and DUOXA2 [*DUOXA2* (MIM *612772)] are essential components for the enzymatic activity of DUOX2 (4) because they form heterodimeric complexes with DUOX2 and allow the translocation of the dimer from endoplasmic reticulum to the membrane (5, 6).

Congenital defects of thyroid hormone synthesis are divided into total iodide organification defect, most frequently due to mutations in the *TPO* gene, which leads to a severe impairment of thyroid function, and partial iodide organification defect (PIOD), generally identified by percentages of discharge after perchlorate test between 10% and 90% and variably associated with alterations in genes such as *PDS*, *Tg*, *DUOX2*, and *DUOXA2* (7–11).

Most of the mutations in *DUOX2* reported either in transient or permanent CH lie in the extracellular portion of the molecule, predicted to interact with TPO, or in the first intracellular loop, adjacent to the calcium binding motifs of the protein. Few data are available to date about the prevalence of *DUOX2* defects, mostly due to the low number of investigated patients, and to the inclusion of *DUOX2* polymorphisms with controversial functional activity (12).

Moreover, the available genetic and clinical data show a lack of genotype to phenotype correlation. Indeed, monoallelic mutations, originally associated with transient cases (13), have also been reported in permanent subclinical CH (14), and even biallelic mutations are associated with extreme clinical variability, ranging from permanent CH (12, 13, 15–18) to transient CH (19–21).

Due to the extreme phenotypic heterogeneity associated with *DUOX2* mutations, clinical features supporting the targeted genetic analysis of *DUOX2* are lacking. Thus, analysis of *DUOX2*, which is particularly money and time consuming, often leads to the identification of wild-type cases, with very low cost-effectiveness.

The aim of the present study was to identify clinical features associated with *DUOX2* mutations. To this purpose, the data available in the literature were evaluated and some enrollment criteria were selected for the population presented here, which is the largest reported to date. Moreover, the in vitro analyses enabled the appropriate classification of novel mutations and other variants with controversial functional impact.

Patients and Methods

Patients

Thirty patients with CH and gland in situ were enrolled starting from August 2011 to December 2012. To be included in the study, patients were required to exhibit a PIOD (discharge rate 10%–90%) or at least two of the following features at diagnosis: 1) elevated serum TSH at confirmatory testing, regardless of blood spot TSH levels; 2) elevated Tg; or 3) hyperplastic thyroid gland at ultrasound. The institutional Ethics Committee approved the study protocol (Grant RF-2010-2309484).

Clinical evaluation

In most cases CH was diagnosed after positive neonatal screening, with a blood spot TSH (bsTSH) threshold level of 10 mU/L. In case of lower bsTSH (7–9.9 mU/L) and the coexistence of risk factors like twin delivery or iodine exposure (eg, cesarean section), a second screening is repeated after 15 days of life. Furthermore, a second screening was performed at 15–30 days of life in all preterm and low-birth weight newborns, and in cases in which there was maternal history of thyroid dysfunction. All patients started L-T_4 treatment for serum TSH (sTSH) levels persistently above 10 mU/L. At diagnosis, thyroid hormones, Tg, and thyroid autoantibodies were measured by commercial kits. Thyroid size was evaluated by ultrasound by measuring the anterior-posterior diameter of the gland. Data on gestational age, mode of delivery, and familial history of thyroid diseases were also collected.

Most patients underwent diagnostic reevaluation at 2–6 years of age, based on thyroid hormone testing after 1 month of L-T_4 withdrawal. Patients showing TSH values persistently higher than the normal range (>5 mU/L) were defined as having permanent thyroid dysfunction. To confirm the diagnosis of transient or permanent CH, our patients are routinely submitted to periodical visits. In particular, in all cases diagnosed as permanent at reevaluation, L-T_4 treatment had to be confirmed on

the basis of the doses required to maintain TSH levels in the normal range. Patients also underwent thyroid ultrasound and ^{123}I scintigraphy with perchlorate ($KClO_4^-$) discharge test to detect iodine organification defects, performed as previously reported (22).

Patients of different ethnicity (Italian, Pakistani, Filipino, Moroccan) were enrolled and none of them suffered from sensorineural hearing loss.

Molecular analysis

Informed consent for DNA analysis was obtained for all screened subjects. All coding regions of *TPO*, *DUOX2*, and *DUOXA2* genes were amplified by PCR using appropriate primers flanking each exon and directly sequenced, as previously reported (10, 13, 23). For *DUOX2* analysis, alternative primers able to discriminate the highly homologous *DUOX2* and *DUOX1* [*DUOX1* (MIM*606758)] coding sequences were also used (Supplemental Figure 1, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). The PCR and sequence of the mutated fragment were repeated at least twice, starting from two separate samples. The DUOX2 variants reported in the present study are indicated according to the systematic nomenclature approved by the Human Genome Variation Society (www.hgvs.org/mutnomen). Nucleotide numbering starts from the A (+1) of the translation initiation ATG codon of the *DUOX2* cDNA sequence reference sequence NM_014080.4 and *DUOX1* cDNA sequence reference sequence NM_017434.3. The amino acid residues of DUOX2 protein are numbered according to the NP_054799.4.

The variants identified were investigated in 50 controls with no documented thyroid disorders. In case of compound heterozygous mutations, the molecular analysis was extended to parents.

Plasmids, cell culture, and functional analysis

For each mutation, a site-directed mutagenesis on the *DUOX2* wild-type (wt) cDNA, previously cloned in the expression vector pcDNA3.1 (24), was performed using an in vitro oligonucleotide mutagenesis system (QuikChange XL site-directed mutagenesis; Stratagene) (primers supplied on request). Briefly, HeLa cells were transiently transfected using 1 μg of total DNA of the constructs carrying the wild-type human *DUOX2* or the variants, together with DUOXA2 expression vector and 3 μL of FuGENE 6 reagent (Roche Applied Science). In particular, 900 ng of each DUOX2 construct were cotransfected with 100 ng of DUOXA2 pcDNA3.1 vector. To monitor the transfection efficiency, a pEGFP vector (CLONTECH Laboratories) was transfected in parallel and green fluorescence protein expression evaluated. Forty-eight hours after transfection, once verified, a transfection efficiency greater than 70%, H_2O_2 generation was measured by reaction with Amplex Red reagent (Invitrogen Life Technologies Corp) producing fluorescent resorfurin in the presence of excess peroxidase, as previously reported (24). The activity of each mutant was expressed as a percentage (mean ± SD) of wild-type activity and corrected for the background obtained in wells transfected with empty vector. Each mutant was analyzed at least in three independent experiments (each performed in duplicate).

RNA extraction and cDNA analysis

RNA was extracted from peripheral leukocytes by means of commercial kits (PAXgene blood RNA system, PreAnalytiX). Total RNA was submitted to reverse transcription with Superscript reverse transcriptase II (Invitrogen Life Technologies) using a random hexamer mixture as primers. Each cDNA was submitted to PCR using two different couples of exonic primers (supplied on request) located 1–3 exons upstream and downstream the mutation site. PCR products were purified and directly sequenced.

Statistical analysis

A Student's *t* test was used for comparisons of the activity of the mutants. Comparison of clinical features between patients and of allelic frequencies of *DUOX2* missense variants between cases and controls were performed using a Mann-Whitney test for nonparametric values or a Fisher's exact test. $P < .05$ was considered statistically significant.

Results

Clinical features and genotype-phenotype correlations

Index cases of the present series

The functional characterization of the *DUOX2* variants demonstrated that 11 of 30 cases (36.7%) harbored a mutation impairing DUOX2 activity (Table 1). In particular, 2 unrelated patients and 2 pairs of siblings had biallelic *DUOX2* mutations, whereas a single heterozygous mutation was detected in 5 cases. The remaining 19 patients were found to harbor silent polymorphisms (Tables 2 and 3 and Figure 1).

The clinical features of the mutant cases were compared with those of patients without *DUOX2* (non-*DUOX2*) pathogenic mutations. At diagnosis, CH cases with *DUOX2* mutations had a more severe phenotype than the non-*DUOX2* patients, with higher Tg (median 655 vs 426 μg/L, range 403–1991 vs 54.4–3000) and lower free T_4 (fT4; 0.7 vs 1 ng/dL, range 0.5–1.4 vs 0.2–1.8) levels, and a higher prevalence of the hyperplastic gland (66.6% vs 29.4%) (data not shown).

Interestingly, at neonatal screening *DUOX2* cases had bsTSH values lower than the non-*DUOX2* patients. In particular, 64% of *DUOX2* were negative at first neonatal screening (bsTSH at 48–72 h of life <10 mU/L), and a second screening was performed just because of prematurity (number 1, number 9), first-degree family history of thyroid dysfunctions (number 4a, number 4b, number 8), or the copresence of borderline bsTSH levels (7–9.9 mU/L) and risk factors (number 6, number 7), as reported in *Patients and Methods*. On the other hand, non-*DUOX2* patients demonstrated a negative first neonatal screening in only 31.5% of cases. An extensive analysis of the initial

Table 1. Clinical Features of Patients With Pathogenic *DUOX2* Mutations

Number	bsTSH at SCR/sTSH at D, mU/L	fT4, ng/dL/Tg, μg/L, at D	US at D APØ, mm	$KClO_4^-$, %	sTSH, mU/L/fT4, ng/dL at RE	Age, mo/L-T4, μg/kg, at RE or Last Visit	Final Diagnosis at RE	DUOX2 Variations	Notes
1	3.5/24.7	0.9/–	10	39	12.1^{OFF}/1.1	45/2.6	P	p.[M866R]; [E1546G]	Cs; 33 GA; Italian[a]
2a	18/180	–/–	–	57	20.4^{OFF}/–	64/2.3	P	p.[Q202Tfs*99]; [T522Pfs*64]	Vd; 40 GA; Italian
2b	21/130	–/–	–	66	23.5^{OFF}/–	36/2.6	P	p.[Q202Tfs*99]; [T522Pfs*64]	Vd; 37 GA; Italian
3	45/>150	–/480	–	–	2.7^{ON}/1.3	72/1.76	(P)	p.[Q570 liter]; [Q570L]	Vd; 38 GA; Pakistani[b]
4a	4.5/31.6	0.7/513	9.4	–	2.8^{OFF}/1.6	24/1.38	T	p.[P966Sfs*29]; [C1052Y]	Vd; 39 GA; Italian
4b	3.1/24.5	0.7/403	7.5	–	1.4^{ON}/–	27/1.6	(T)	p.[P966Sfs*29]; [C1052Y]	Vd; 37 GA; Italian
5	12/31.9	1.5/736	8	15	6.6^{OFF}/1.3	42/1.3	P	p.[P966Sfs*29]	Cs; 40 GA; Italian
6	8.9/100	0.5/–	>9	–	4.5^{ON}/–	22/2.3	(P)	p. [P966Sfs*29]; [*P303R*]	Cs; 38 GA; Italian
7	8.8/23.7	0.7/655	11.1	–	7.2^{ON}/–	46/1.9	(P)	p.[P966Sfs*29]	Cs; 40 GA; Italian
8	6.3/101	0.6/1991	9.9	–	3.1^{ON}/–	14/3.15	(P)	c.[2335-1G>C]	Cs; 38 GA; Italian
9	6/353	1.4/–	>9	–	5.2^{ON}/–	33/3.3	(P)	p.[P341S]; [*P138L;L171P*]	Cs; 30 GA; Italian

Abbreviations: APØ, antero-posterior diameter; cs, caesarean section; D, diagnosis; GA, gestational age; $KClO_4^-$, perclorate discharge rate; –, not done; N, normal; OFF, L-thyroxine withdrawal; ON, on L-thyroxine; P, permanent (letters in parentheses indicate that the outcome has been established based on TSH levels and L-thyroxine requirement at the last visit); RE, reevaluation; SCR, screening; s, serum; T, transient (letters in parentheses indicate that the outcome has been established based on TSH levels and L-thyroxine requirement at the last visit); US, ultrasound scan; vd, vaginal delivery. Nonpathogenic DUOX2 variations are reported in *italics*. p.P138L and p.S1067L variants are in homozygosis if not reported in the table. Reference ranges are as follows: TSH at diagnosis, 0.5–6.3 mU/L; fT4 at diagnosis, 1.5–2.4 ng/dL; Tg, 10–150 μg/L; ultrasound scan at diagnosis, less than 9 mL; $KClO_4^-$, less than 10%; TSH at reevaluation, 0.25–5 mU/L; fT4 at reevaluation, 0.7–1.7 ng/dL.

[a] Dizygotic twin from intracytoplasmic sperm injection.

[b] Consanguineous.

TSH trend in *DUOX2* patients revealed in all cases a confirmatory sTSH level that was higher than the normal limit (median bsTSH 8.8 mU/L, median sTSH 100). In contrast, a similar TSH trend could be found in only 2 of 19 patients without *DUOX2* mutations, most them showing stable or only slightly increased sTSH values (median bsTSH 11.8 mU/L, median sTSH 26.6). The median δ-increase (sTSH/bsTSH) was significantly different between *DUOX2* and non-*DUOX2* cases (605.7% vs 148.7%, $P < .05$ by Fisher's exact test).

At reevaluation, Tg was normal and thyroid ultrasound scan documented a normal gland in all patients, as a likely consequence of treatment. ^{123}I scintigraphy with perchlorate discharge test was performed in 19 of 30 cases and

Table 2. Clinical Features of Patients Without Pathogenic *DUOX2* Mutations

Number	bsTSH at SCR/sTSH at D, mU/L	fT4, ng/dL/Tg, μg/L at D	US at D APØ, mm	$KClO_4^-$, %	sTSH, mU/L/fT4, ng/dL, at RE	Age, mo/L-T4, μg/kg, at RE or Last Visit	Final Diagnosis at RE	DUOX2 Variations	Notes
10	2.3/18.2	1.1/–	>10	14	11.3^{OFF}/1.2	59/1.6	P	wt	Cs; 40 GA; Italian
11	5/13.3	1.5/54.4	6	30	11.7^{OFF}/1.1	48/2.36	P	wt	Vd; 38 GA; Italian
12	12.4/17.2	1.1/146	7.5	30	13.5^{OFF}/0.9	48/2	P	wt	Cs; 36 GA; Italian
13	13.3/37.4	1/365	9	34	6^{OFF}/1.3	36/1.8	P	wt	Cs; 40 GA; Italian
14	11.3/19.8	1.3/488	7.5	28	6.2^{OFF}/1.2	43/1.8	P	wt	Vd; 40 GA; Italian
15	5.1/11.8	1/58.2	7	15	20^{OFF}/0.9	36/2.6	P	wt	Cs; 32 GA; Italian[a]
16	31.4/218	0.6/–	8.8	15	1634^{OFF}/0.5	40/3.99	P	p.[*P303R*]	Vd; 39 GA; Italian
17	28.7/33.4	0.7/1620	8	26	6.6^{OFF}/1	47/1.9	P	wt	Cs; 33 GA; Italian
18	N/9[b]	–/–	–	62	9.3^{OFF}/1.1	72/1.24	P	wt	Vd; 40 GA; Italian
19	15/9.3	1.6/–	5	19	10.6^{OFF}/0.9	72/1.5	P	wt	Cs; 40 GA; Italian
20	10/12.1	1/–	6.1	10	22.7^{OFF}/0.9	55/2.6	P	wt	Vd; 40 GA; Italian
21	10.7/11.5	1.8/56	7	51	2.5^{OFF}/1.4	48/0.99	T	wt	Vd; 38 GA; Italian
22	4.4/158	0.6/–	N	45	1.2^{OFF}/1.2	54/0.65	T	wt	Cs; 28 GA; Filipino
23	10/44.8	1/280	N	15	3.1^{OFF}/1	57/1.5	T	p.[*P138L*; *S1067L*]; [*H678R*; *R701Q*; *P982A*]	Cs; 40 GA; Italian
24	22/–	–/–	–	27	1^{OFF}/27	53/1.13	T	wt	Cs; 37 GA; Italian
25	25/56.8	0.6/493	11.5	–	5.9^{ON}/–	26/3	(P)	p.[*S1067L*]	Vd; 40 GA; Moroccan
26	8.5/38.1	0.9/1202	9	–	1.7^{ON}/–	28/2.14	–	wt	Cs; 39 GA; Italian
27	14.5/123.2	0.6/3000	9.6	–	0.7^{ON}/–	19/1.9	–	p.[*S1067L*]	Vd; 41 GA; Moroccan
28	100/208	0.2/3000	8.2	–	2.1^{ON}/–	36/3.87	(P)	wt	Vd; 40 GA; Italian

Abbreviations: APØ, antero-posterior diameter; cs, caesarean section; D, diagnosis; GA, gestational age; $KClO_4^-$, perclorate discharge rate; –, not done; N, normal; OFF, L-thyroxine withdrawal; ON, on L-thyroxine; P, permanent (letters in parentheses indicate that the outcome has been established based on TSH levels and L-thyroxine requirement at the last visit); RE, reevaluation; SCR, screening; s, serum; T, transient (letters in parentheses indicate that the outcome has been established based on TSH levels and L-thyroxine requirement at the last visit); US, ultrasound scan; vd, vaginal delivery. Nonpathogenic DUOX2 variations are reported in *italics*. p.P138L and p.S1067L variants are in homozygosis if not reported in the table. Reference ranges are as follows: TSH at diagnosis, 0.5–6.3 mU/L; fT4 at diagnosis, 1.5–2.4 ng/dL; Tg, 10–150 μg/L; ultrasound scan at diagnosis, less than 9 mL; $KClO_4^-$, less than 10%; TSH at reevaluation, 0.25–5 mU/L; fT4 at reevaluation, 0.7–1.7 ng/dL.

[a] Urinary iodine at birth: 3340 μg/L.

[b] Incidental detection at 3 years old.

Table 3. Allelic Frequency of *DUOX2* Missense Variations in CH Patients and Controls and Relative H_2O_2 Production

Variant	Reference SNP	Reference	CH	Haplotype (Protein)	wt Activity, %	AF		Statistics	
						CH	Controls	*P*	OR (95% CI)
c.413C>T p.P138L	2001616	1000 Genomes	–	–	–	–	0.219	–	–
		(12)	–	–	–	0.548	0.042	.0001	27.93 (12.46–62.6)
		Present study	–	All CH cases	122	0.967	0.67	<.0001	14.28 (3.28–62.14)
c.512T>C p.L171P	199957468	1000 genomes	–	–	–	–	0.013	–	–
		Present study	(P)	p.[P341S];[P138L; L171P]	90	0.016	0.048	.41	–
c.908C>G p.P303R	151261408	1000 genomes	–	–	–	–	0.005	–	–
		Present study	P (P)	p.[P303R], p.[P966Sfs*29];[P303R]	75	0.033	0.06	.71	–
c.1021C>T p.P341S	–	Present study	(P)	p.[P341S];[P138L; L171P]	61	0.016	0	.37	–
c.1709A>T p.Q570L	–	Present study	(P)	p.[Q570L];[Q570L]	25	0.033	0	.14	–
c.2033A>G p.H678R	57659670	1000 genomes	–	–	–	–	0.150	–	–
		(19)	T	p.[H678R];[S1067L]	–	0.125	0	.07	–
		(14)	P	p.[H678R; R701Q; P982A]	85	0.045	0.06	1	–
		(12)	P	p.[H678R];[L716WfsX34]	85	0.103	0.035	.01	3.6 (1.31–7.62)
			P	p.[H678R];[H678R]					
			P	p.[H678R];[H678R]					
			P	p.[H678R];[I1080T]					
		(25)	T	p.[E327X];[H678R]	–	–	–	–	–
		Present study	T	p.[P138L; S1067L]; [H678R; R701Q; P982A]	99	0.016	0.02	1	–
c.2102G>A p.R701Q	201109959	1000 genomes	–	–	–	–	0.031	–	–
		(14)	P	p.[H678R; R701Q; P982A]	83	0.045	0.06	1	–
		Present study	T	p.[P138L; S1067L]; [H678R; R701Q; P982A]	84	0.016	0.02	1	–
c.2597T>G p.M866R	200948626	Present study	P	p.[M866R];[E1546G]	0	0.016	0	.37	–
c.2944C>G p.P982A	61730030	1000 genomes	–	–	–	–	0.029	–	–
		(14)	P	p.[P982A] p.[H678R; R701Q; P982A]	96	0.09	0	.02	29.39 (1.36–635)
		Present study	T	p.[P138L; S1067L]; [H678R; R701Q; P982A]	99	0.016	0.02	1	–
		1000 genomes	–	–	–	–	0.002	–	–
c.3155G>A	76343591	(18)	P	p.[S911L];[C1052Y]	72	–	0	–	–
p.C1052Y		Present study	T	p.[P966Sfs*29];[C1052Y]	17	0.033	0	.14	–
			(T)	p.[P966Sfs*29];[C1052Y]					
c.3200C>T, p.S1067L	269868	1000 genomes	–	–	–	–	0.245	–	–
		(19)	T	p.[H678R; S1067L]	–	0.25	0.01	.014	33 (2.61–417.8)
			T	p.[K530X];[E879K; S1067L]					
			T	p.[K530X];[E879K; S1067L]					
		(12)	–	–	–	0.142	0.058	.0017	2.67 (1.47–4.86)
		Present study		All CH cases	122	0.95	0.83	.74	–
c.4637A>G, p.E1546G	201229193	Present study	P	p.[M866R];[E1546G]	54	0.016	0	.37	–

Abbreviations: AF, allelic frequency; CI, confidence interval; –, not done; OR, odds ratio; P, permanent (letters into parentheses indicate that the outcome has been established based on TSH levels and L-thyroxine requirement at the last visit); T, transient (letters into parentheses indicate that the outcome has been established based on TSH levels and L-thyroxine requirement at the last visit). 1000 Genomes can be found at www.1000genomes.org.

revealed a partial iodine organification defect (discharge rate of 10%–66%) in all cases. Discharge rate at reevaluation was higher in *DUOX2* patients (median 48%, range 15–66%) with respect to non-*DUOX2* cases (median 27%, range 10–62%). Based on the reevaluation assessment, or considering the TSH levels and the L-T_4 requirement at the most recent visit, CH has been classified as permanent in 22 of 30 (nine *DUOX2* cases) and transient in 6 of 30 cases (two *DUOX2* cases).

Among *DUOX2* patients, no significant differences were noted between monoallelic and biallelic cases concerning bsTSH, sTSH, fT4, Tg, goiter prevalence, discharge rates at diagnosis, or reevaluation and outcome of the CH (data not shown).

Index cases from all the reported series

Due to the interesting trends found in our series, the clinical features of our cases were pooled with those described for all the cases with and without *DUOX2* mutations reported so far. In Supplemental Table 1, all the *DUOX2*-related CH cases reported to date (n = 40) are shown. Biallelic mutations were reported in 22 cases (16

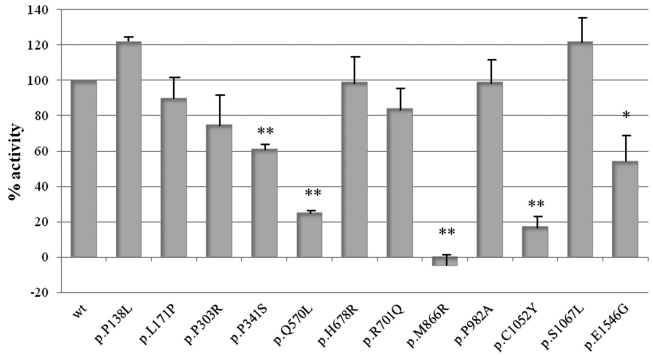

Figure 1. H_2O_2 generation of DUOX2 mutants was measured with Amplex Red reagent and expressed as percentage (mean ± SD) of wild-type activity. The statistical significance with respect to the wild-type activity is also reported. *, $P < .05$, **, $P < .01$ by Student's *t* test.

families), whereas 13 cases had monoallelic mutations. In the remaining five patients, nonsynonymous missense variants were the only variants found, which were classified as nondeleterious single-nucleotide polymorphisms (SNPs) in the present paper.

In *DUOX2* patients we found sTSH (median 100 mU/L, range 8–1400 mU/L) and serum fT4 (sfT4; median 0.6 ng/dL, range 0.07–1.86 ng/dL) at diagnosis that were significantly higher and lower, respectively, than in the non-*DUOX2* cases (median sTSH 35.4 mU/L, range 6–218 mU/L; sfT4 1 ng/dL, range 0.2–1.8 ng/dL) (Figure 2), and the combination of sTSH and sfT4 levels showed a tendency to the separation of the mutated from the nonmutated cases (Supplemental Figure 2A). Interestingly, the fT4 to TSH ratio was significantly lower in *DUOX2* patients and the receiver-operating characteristic curve analysis identified 0.0584 or less as the most accurate fT4/TSH cutoff value to predict *DUOX2* status (sensitivity 94.9, specificity 40, area under the curve 0.686, $P = .014$) (Supplemental Figure 2B). Although Tg values were overlapping among the two groups, the *DUOX2* cases showed a higher median (800 ng/mL, range 40–4496 ng/mL) than the non-*DUOX2* patients (490.5 ng/mL, range 54.4–3000 ng/mL). Moreover, goiter was present in a higher number of *DUOX2* patients (19 of 29, 65.5%) than in non-*DUOX2* cases (5 of 19, 26.3%) ($P < .05$, by Fisher's exact test, data not shown), whereas no significant differences were found in the discharge rate at re-evaluation (median in *DUOX2* cases: 33.5%, range 0–100 vs median in non-*DUOX2* cases: 22.5%, range 0–99).

Molecular and functional characterization of DUOX2 variants

Description

The analysis of *DUOX2* coding sequence led to the identification of two previously reported (c.602dup, p.Q202Tfs*99, found in the literature as ins602gfsX300, and c.2895_2898del, p.P966Sfs*29, found in the literature as S965fsX994) (13, 14, 16, 17) and one novel

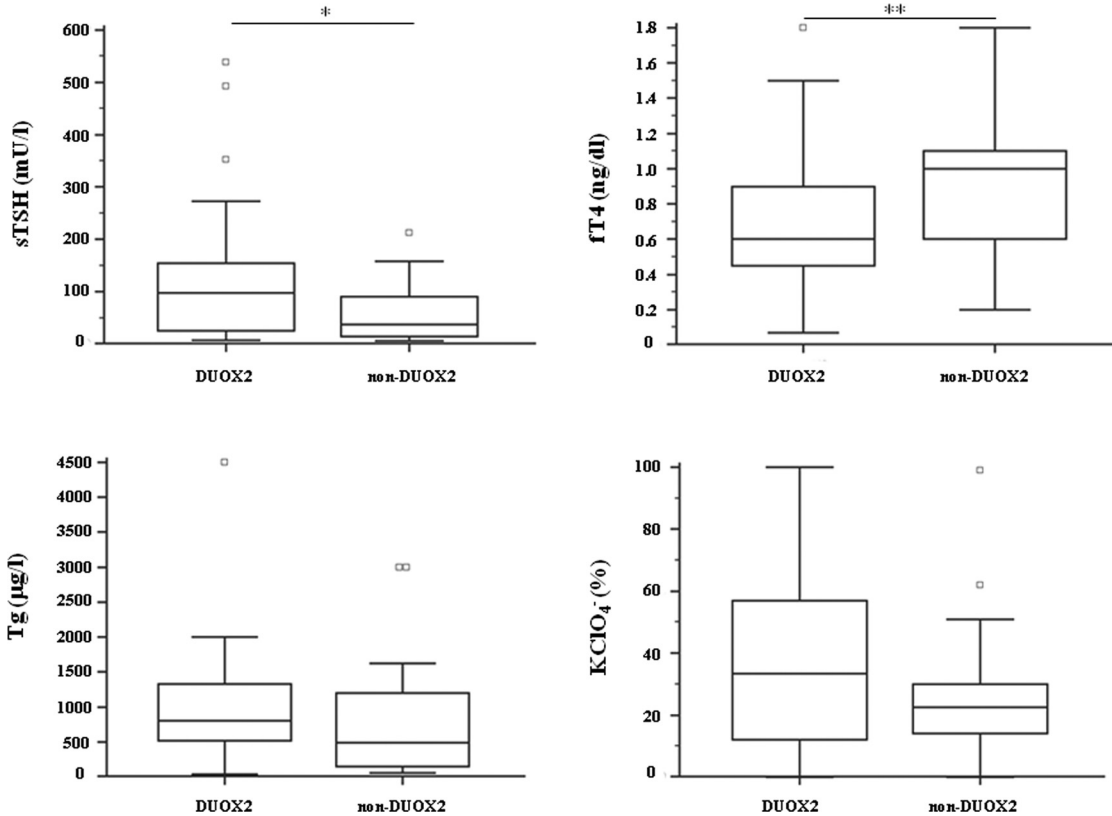

Figure 2. The most relevant clinical features in patients with CH and mutations in the *DUOX2* gene reported so far including the present paper (DUOX2) were compared with data of CH patients without *DUOX2* mutations (non-DUOX2). *, $P < .05$, **, $P < .01$ by Mann-Whitney test.

(c.1564del, p.T522Pfs*64) frameshift mutations and one novel splice site mutation (c.2335-1G>C) (Table 1). These variants were not identified in 50 controls (100 alleles). In particular, the deletion c.2895_2898del, p.P966Sfs*29, was the most frequent mutation found in our series, detected in 4 of 28 unrelated patients (14%). Interestingly, the c.602dup, p.Q202Tfs*99 (number 2A and number 2B in Table 1) was detected only by the use of *DUOX2*-specific primers, able to discriminate the highly homologous *DUOX2* and *DUOX1* coding sequences (Supplemental Figure 1).

In addition, 12 missense variants were found (Tables 1, 2, and 3). Six of these (c.413C>T, p.P138L; c.2033A>G, H678R; c.2102G>A, p.R701Q; c.2944C>G, p.P982A; c.3155G>A, p.C1052Y; and c.3200C>T, p.S1067L) have been already reported in patients with CH, and for some of them, the definite role as pathogenic mutants or SNPs is debated (12, 14, 18, 19, 25). The remaining six missense *DUOX2* variants (c.512T>C, p.L171P; c.908C>G, p.P303R; c.1021C>T, p.P341S; c.1709A>T, p.Q570L; c.2597T>G, p.M866R; and c.4637A>G, p.E1546G) have not been previously found in CH cases. Because some nonsynonymous SNPs are probably neither functional nor enriched in CH cases, we investigated their allelic frequency in controls and performed accurate functional analyses (Figure 1). In particular, p.P138L, p.L171P, p.P303R, p.H678R, p.R701Q, p.P982A, and p.S1067L variants were found in normal controls (Table 3).

Mutations in the *DUOXA2* gene were always screened for, whereas *TPO* was studied only in cases without or with monoallelic mutation in *DUOX2* and/or with perchlorate discharge levels greater than 70%, and/or serum TSH levels at diagnosis greater than 100 mU/L. No mutation was detected in either then *DUOXA2* or *TPO* genes.

Functional analysis

The functionality of the 12 *DUOX2* missense variants was tested after expression in HeLa cells. As shown in Figure 1, four mutants (p.P341S, p.Q570L, p.C1052Y, and p.E1546G) displayed a significantly reduced of H_2O_2 generation activity (17%–61% of wt protein, $P < .05$) whereas a complete loss of function was recorded with plasmids carrying the p.M866R mutation ($P < .01$). These five variants were thus considered as deleterious. The H_2O_2 generation activity of the remaining variants was instead similar to that of the wt protein.

Thus, considering both the allelic frequencies data (Table 3) and the functionality (Figure 1) of the remaining seven missense variants, the p.P138L appears functionally silent but significantly associated with CH in Japan and Italy ($P = .0001$), whereas p.H678R and p.S1067L are associated with CH only in the Japanese population ($P = .01$, $P = .01$). The remaining four missense variants were neither enriched in CH cases nor functionally active.

cDNA analysis

To study the effect of the stop-codon and the splice site mutations, the cDNA retrotranscribed from the peripheral leukocytes mRNAs of the patients was amplified using two different pairs of primers.

In case 5, harboring the c.2895_2898del, p.P966Sfs*29 mutation at the DNA level, the analysis of the retrotranscribed cDNA led to the amplification of fragments of expected size. However, direct sequencing of these PCR products revealed only the wild-type allele, indicating the degradation of the mutated allele at the mRNA level (Figure 3A).

In the genomic DNA of patients number 2A and number 2B, different frameshift mutations were found: one inherited from the father (c.1564del, p.T522Pfs*64) and one of maternal origin (c.602dup, p.Q202Tfs*99). The analysis of c.1564del at the cDNA level of patient number 2B resulted in the amplification of both paternal mutated and wt alleles (Figure 3B). On the other hand, the analysis of c.602dup showed the skipping of exon 5 (Figure 3C). This phenomenon, which has been previously reported in a CH patient (16), is demonstrated for the first time in this study to occur in the cDNA from lymphocytes of control subjects and in other tissues (thyroid, testis, pituitary, orbital fat), indicating the existence of a physiologically occurring alternative splicing product (data not shown). The direct sequencing of the correctly spliced exon 5 in patient 2B revealed only the wt allele, suggesting the skipping or the degradation of the mutated maternal allele (Figure 3C).

The splice-site mutation c.2335-1G>C, identified in patient number 8 is predicted to lead to the skipping of exon 18 or, alternatively, to activate a cryptic acceptor site in intron 17 or exon 18 (www.umd.be/hsf). To test these possibilities, we performed different RT-PCRs spanning exons 17–19, and we detected only bands of the expected size, confirmed by direct sequencing (data not shown). A possibility exists that the alternatively spliced allele could be submitted to degradation; however, because patient 8 did not harbor any informative SNPs in the *DUOX2* gene, we were not able to test this hypothesis.

Discussion

In recent years, *DUOX2* emerged as a relevant gene among those involved in dyshormonogenesis. Nevertheless, due to the relevant intra- and interfamilial phenotype

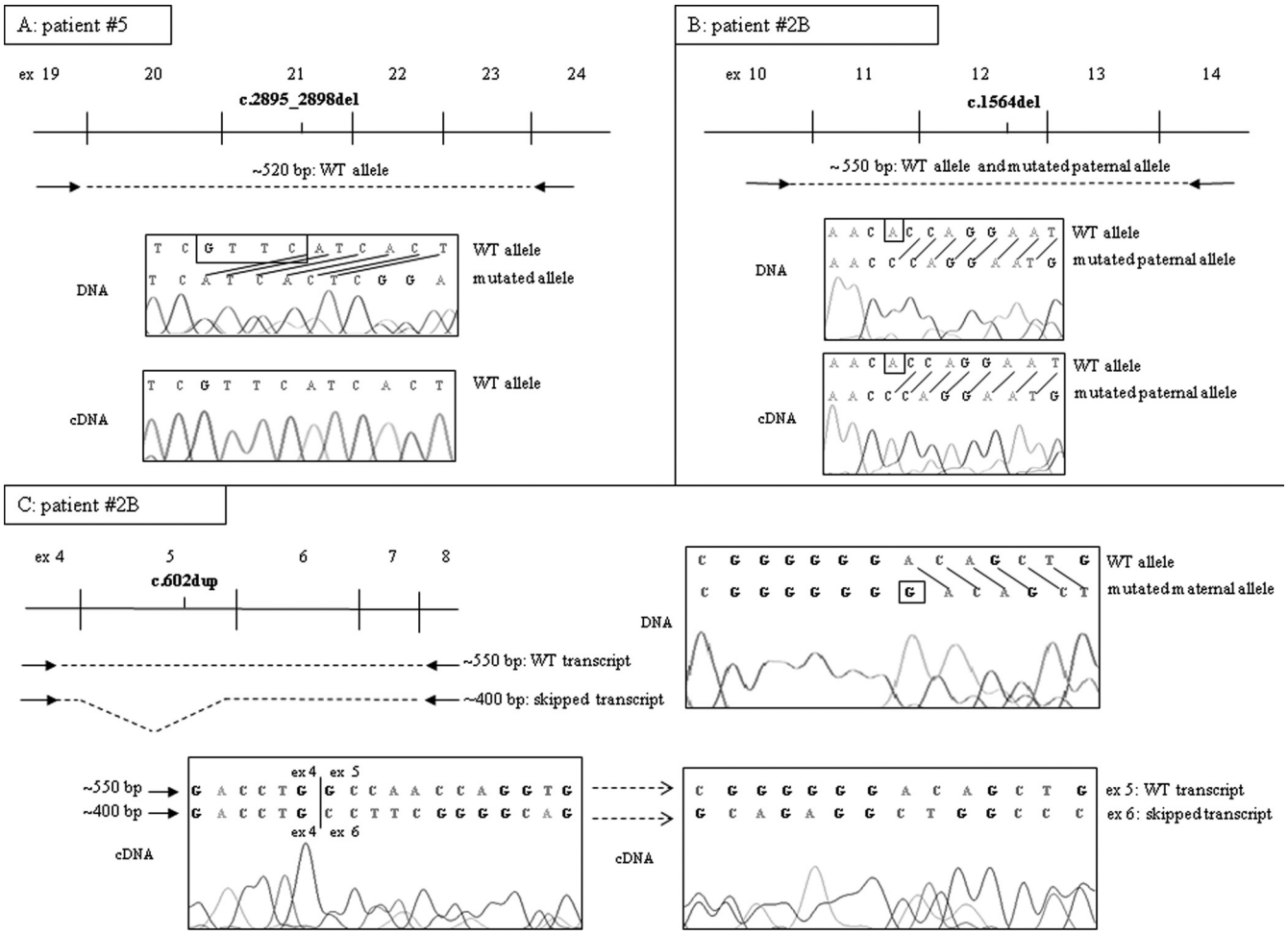

Figure 3. Exemplificative cases of amplification of cDNA from lymphocytes in patients number 5 (c.2895_2898del) and number 2B (c.1564del and c.602dup). The position of the mutations on the transcript is schematically represented. The sequencing chromatograms of DNA and cDNA are reported and the mutations are indicated by boxes. The position of the RT-PCR primers (arrows) and the RT-PCR products (dashed lines) are also shown. A, In patient 5, the wt transcript was the only one detected, as shown by the comparison with the DNA sequence. B, In patient 2B, the c.1564del mutation was detected both at the DNA and cDNA levels. C, In patient 2B, the c.602dup mutation was documented at the DNA level (seven Gs instead of six Gs). An alternative splicing product, lacking exon 5, was observed at the cDNA level. The direct sequencing of the correctly spliced exon 5 in patient 2B revealed only the wt allele, suggesting the skipping or the degradation of the mutated maternal allele.

variability associated with monoallelic and biallelic mutations and to the limited number of patients included in different series, a reliable genotype-phenotype correlation has not been established to date. Thus, the aim of the present paper was to search for and characterize *DUOX2* mutations in a large series of 26 unrelated children and two pairs of siblings selected by apparently stringent criteria consistent with the presence of a PIOD. We detected 16 different *DUOX2* variations (three frameshift, one splice site, and 12 missense). Functional studies performed to clarify the pathogenic impact of the missense variants, classified seven of them as nonsynonymous single-nucleotide variations, consistent with their detection in normal controls. In contrast, five missense variants were absent in normal controls and showed a variable but significantly reduced H_2O_2 generation capacity, together with a high degree of conservation of the affected residues across animal species, suggesting their causative role in the pathogenesis of CH. Thus, in this selected population, the prevalence of *DUOX2* pathogenic mutations was 36.7%.

The frameshift p.P966Sfs*29, commonly reported as p.S965fsX994, was the most frequent *DUOX2* mutations detected in our series, consistent with data in the literature. Here we show that the transcripts carrying this deletion are submitted to nonsense mRNA decay, indicating the existence of mRNA quality control active on the *DUOX2* gene. A more complex mechanism has been hypothesized for the mutation p.Q202Tfs*99 found in two siblings and lying in exon 5. Indeed, after demonstrating that an alternative splicing product lacking exon 5 exists in normal tissues from different organs, we hypothesized the skipping or the degradation of the mutated transcript. Concerning the novel mutation p.T522Pfs*64, which is located in exon 12 and generates a premature stop codon in exon 14, we showed that the pathogenic effect is likely due

to the truncation of the protein and not to mRNA degradation.

The prevalence of *DUOX2* mutations found in the present cohort was consistent with data of the literature (30%–45%) (12–14, 17), but the mutation rate is expected to largely vary among different series depending on the criteria used for enrollment. Thus, with the aim to identify more selective and stringent clinical parameters associated with *DUOX2* mutations, we compared *DUOX2* and non-*DUOX2* cases, pooling the data from the present series and those available in the literature. These data showed that *DUOX2* patients had, at diagnosis, significantly higher serum TSH and lower fT4 levels and a higher prevalence of goiter than the non-*DUOX2* cases. Consistently, the fT4 to TSH ratio was significantly lower in the *DUOX2* cases ($P = .01$), and the receiver-operating characteristic curve analysis identified the fT4/TSH cutoff value with the highest accuracy for the prediction of *DUOX2* status (≤ 0.058).

Moreover, although not statistically significant, Tg levels and discharge rates after perchlorate were higher in the *DUOX2* cases. The bsTSH levels represent another critical finding because they were found to be lower than the referral limit of 10 mU/L in 7 of 11 *DUOX2* and in 6 of 19 non-*DUOX2* cases, indicating the importance of serum TSH measurement, at least in premature newborns or in children with a familial thyroid disorder or with bsTSH levels greater than 7 mU/L, as suggested by the guidelines of our Italian region (26). According to reevaluation or considering the L-T_4 requirement at the last visit, a permanent congenital hypothyroidism was diagnosed in four of six cases with biallelic *DUOX2* mutations and in five of five monoallelic cases. No correlations were noted between the type of *DUOX2* mutation (missense, stop codon, splice site, monoallelic or biallelic, H_2O_2 generation activity) and the severity or duration of the CH, consistent with data in the literature (11).

Some hypotheses have been drawn to explain the interfamilial variability of the DUOX2 phenotype. In particular, the existence of other H_2O_2 generating systems, such as DUOX1/DUOXA1, or alternatively the age variability in thyroid hormone requirements may account for transient biallelic cases. On the other hand, permanent CH cases with monoallelic or without mutations could be explained by cryptic *DUOX2* variations, such as large rearrangements not detectable at standard genetic techniques, the concurrent involvement of multiple gene defects, or epigenetic mechanisms or environmental factors. Finally, the high homology of *DUOX2* and *DUOX1* coding sequences could represent a methodological problem. In this context, we are the first to demonstrate that mutations can be missed when using primers not exclusively specific for *DUOX2* sequence.

In conclusion, 11 of 30 patients of our selected series (36.7%) were found to harbor a mutation functionally impairing DUOX2 activity, indicating that alterations in the peroxide generation system account for a large portion of CH cases with PIOD. Based on our results and pooling all the data of the literature, we found that neither Tg levels nor discharge rates after perchlorate nor the presence of goiter were able to distinguish carriers from noncarriers. On the other hand, we found that a low fT4 to TSH ratio may be more accurate in this prediction.

Acknowledgments

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