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Research Article

Biallelic Stop Codon Mutations (p.F353Pfs*36/p.Y425X) in *DUOX2* Gene Associated with Transient Congenital Hypothyroidism: Report of a Family and Literature Review

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Abstract

Purpose: DUOX2 deficiency is a transient or permanent disorder that results in thyroid dyshormonogenesis. The purpose of this study was to identify and characterize new mutations in the *DUOX2* gene in an attempt to increase the understanding of genotype-phenotype correlation for this disorder. The current study summarizes also the spectrum of *DUOX2* variations reported to date in the literature.

Methods: Two siblings from an nonconsanguineous family with clinical and biochemical criteria suggestive of transient CH were studied. Single-Strand Conformation Polymorphism (SSCP) analysis and sequencing of DNA of *TPO* and *DUOX2* genes were performed.

Results: Sequencing analysis of *DUOX2* gene revealed two inactivating mutations, a novel c.1057_1058delTT mutation (p.F353Pfs*36, father's mutation) and a possible previously reported c.1275T>G mutation (p.Y425X, mother's mutation). Consequently, the two siblings carry a compound heterozygous for p.F353Pfs*36/ p.Y425X mutations, whereas the healthy brother is heterozygous for the c.1275T>G mutation and does not carry the c.1057_1058delTT mutation. Up to date, hundred twenty pathogenic variations and functional single nucleotide polymorphisms in the human *DUOX2* gene have been reported associated with transient or permanent CH: 78 missense mutations, 11 nonsense mutations, 26 deletions and insertions, and 6 splice site mutations. The transient or persistent variability of the CH phenotype is not directly related to the number of mutant *DUOX2* alleles. Pathogenic *DUOX2* mutations were identified together with likely pathogenic variants in the *TSHR*, *DUOXA2*, *Thyroid peroxidase*, *Thyroglobulin* and *SLC26A4* genes.

Conclusion: In the present study, we have identified a novel p.F353Pfs*36 mutation in peroxidase like domain of DUOX2 and we have confirmed that total loss of DUOX2 activity by biallelic premature termination codon causes transient CH phenotype.

Keywords: Congenital hypothyroidism; DUOX2 gene; Mutation; Compound heterozygous mutations; Premature stop codon

Introduction

Iodide Organification Defects (IOD) associated with mutations in the *TPO* (Thyroid Peroxidase), *SLC26A4* (solute carrier family 26 member 4, pendrin), *DUOX2* (Dual Oxidasa 2) or *DUOXA2* (DUOX maturation factor 2) genes are implicated in the pathogenesis of Congenital Hypothyroidism (CH) [1]. IOD is characterized by high levels of serum TG and TSH with simultaneous low levels of circulating thyroid hormones and a positive Perchlorate Descarge Test (PDT), indicating that the iodide is taken up by thyroid cells but it is not incorporated into the TG protein [1,2].

The key enzymatic system involved in the iodide organification process is located on the apical plasma membrane of polarized follicular thyroid cells. TPO catalyzes both the iodination and coupling of hormonogenic tyrosyl residues of TG with an absolute requirement of hydrogen peroxide, which acts as an electron acceptor [3]. H_2O_2 is generated by a metabolic pathway, involving DUOX1 and DUOX2 [3-5].

PDT is used to distinguish Total Iodide Organification Defect (TIOD) from Partial Thyroid Organification Defect (PIOD). *TPO* gene mutations in patients with PIOD usually affect a single allele, whereas homozygous or compound-heterozygous *TPO* mutations are associated to TIOD [1]. Mutations in *SLC26A4* gene cause Pendred syndrome characterized by congenital sensorineural hearing loss, goiter with or without hypothyroidism and usually PIOD [1]. Most of patients with mutations in *DUOX2* gene are characterized by PIOD. Furthermore, mutations in *DUOX2* gene have been associated to

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Table 1: Laboratory data of patients with transient congenital hypothyroidism and DUOX2 defects.

Relative	Age	TSH mUI/L	TT4 µg/dl	FT4 ng/dl	TT3 ng/dl	TG ng/ml	Anti-TPO /Anti-TG U/ml
I-1		1.91	6.7	1.13	118	9.1	<10/<20
I-2		1.23	7.7	1.19	113	10	<10/<20
II-1	Screening at 2 days	32.5 (blood)					
	1 month evaluation	32	13	1.46	ND	266	<10/<20
	2.72 years Re-evaluation*	5.22	8.2	1.17	ND	41.7	<10/<20
	7 years Re-evaluation*	4.8	7.2	1.7	145	51.7	<10/<20
	11 years evaluation	2.4	7.0	1.06	154	41.0	<10/<20
II-2		2.60	9.5	1.15	182	10.9	<10/<20
II-3	Screening at 2 days	19 (blood)					
	15 days evaluation	33	7.9	0.92	260	666	<10/<20
	3.32 years Re-evaluation *	4.87	10.2	1.37	173	41.7	<10/<20
	7 years evaluation	2.06	8.6	1.27	126	17.6	<10/<20
Reference range		<60 days: 1.6-10, 2-12 months: 1-6.8, > 1 year: 0.5–6.5	<30 days: 6–18, >1 month: 6-14, > 17 years: 4.5–12.5	<30 days: 1–2.6, >1 month: 0.8–2.2, > 17 years: 0.8-2.0	<30 days: 80-260, 1-12 months: 80-245, > 1 year 80-220	< 30 days: 30-100, >1 month 6-40	<17 years: <20, Adults <35

* Data were obtained after one month of levothyroxine withdrawal.

transient CH (TCH) or permanent CH (PCH), with a high intra and interfamilial phenotypic variability [2]. Instead, TCH can be caused by iodine excess or deficiency and exposure to maternal antithyroid drugs or transplacental antibodies [6,7]. PCH requires lifelong thyroid hormone replacement.

Most cases of CH associated with alterations in the DUOX2 are caused by either biallelic or monoallelic mutations which lead to relantionships between DUOX2 genotypes and clinical phenotypes extremaly complex [8-10]. Both biallelic and monoallelic DUOX2 mutations could be associated with TCH or PCH [11,12]. Because *DUOX2* biallelic mutations commonly are inherited in an autosomal recessive manner, the patients should be homozygous or compound heterozygous for gene mutations and the parents should be carriers of one *DUOX2* mutation. Up to date, 121 variations have been identified and characterized throughout the DUOX2 gene [8,9,11-13-47].

In the present study we report a family with transient CH. Screening of *DUOX2* gene revealed two inactivating mutations, a novel c.1057_1058delTT mutation (p.F353Pfs*36) and a possible previously documented c.1275T>G mutation (p.Y425X) [19], conforming a new compound heterozygous. Remarkable, as our findings confirm, severe biallelic defects of *DUOX2* in transient CH infers compensatory mechanisms in the peroxide supply.

Materials and Methods

Patients

We report 2 siblings of nonconsanguineous healthy parents and affected with congenital hypothyroidism which led to perform genetic testing.

The proband (II-1), born in 2003, was a girl who was detected through neonatal screening with elevated TSH, 32.5 mUI/l blood at 2 days of life (Cut of <10 mUI/L). Thyroid profile at 1 month of age showed TSH: 32 mU/L (reference range: <10), TT4 13 ug/dl, (reference range 6-18) FT4: 1.46 ng/dl (reference range:1-2.6) and TG: 266 ng/dl (reference range: 30-100) with no circulating autoantibodies (anti-TPO and anti-TG) (Table 1) and eutopic enlarged thyroid gland in the ^{99m}Tc scan. These results suggested a defect in thyroid hormone synthesis and so treatment with levothyroxine was started at 25 µg per day and continued since 2.9 years of age. At that time treatment was discontinued for a month in order to reevaluate her thyroid function. Serum TSH, TT_4 and FT_4 levels were normal and TG slightly elevated (Table 1). A Perchlorate discharge test was performed with 17% of discharge (normal <15%). Thyroid ultrasound was normal. Treatment was restarted and stopped again for a month at 7 years of age. Thyroid profile was normal (Table 1), perchlorate test negative and TG: 51.2 ng/dl (reference range: 6-40). Therefore levothyroxine replacement was not necessary. She is now 12 years old, grows normally, has an adequate school performance, undergoes normal puberty and keeps euthyroid.

Her brother (II-3), born in 2007, was also detected through neonatal screening, 19 mUI/L at 2 days of life. Thyroid profile at 15 days showed TSH: 33 mU/L, T_4: 7.9 µg/dl, FT4: 0.9 ng/dl and TG: 666 ng/dl (Table 1). He started treatment with levothyroxine 25 µg/day, grew and developed normally and was reevaluated with treatment withdrawal for a month at 3.72 years of age. His thyroid function was normal excepting a slightly high TG (Table 1), thyroid gland was normal on ultrasound and the perchlorate discharge test negative. The treatment was stopped. He grew in the 50 th percentile and has a normal high Intelligence Quotient (IQ) at age 6. With 7 years of age he continues euthyroid and thyroglobulin levels have normalized.

The father (I-1), mother (I-2) and the healthy brother (II-2) of the proband had normal thyroid function (Table 1) without symptoms

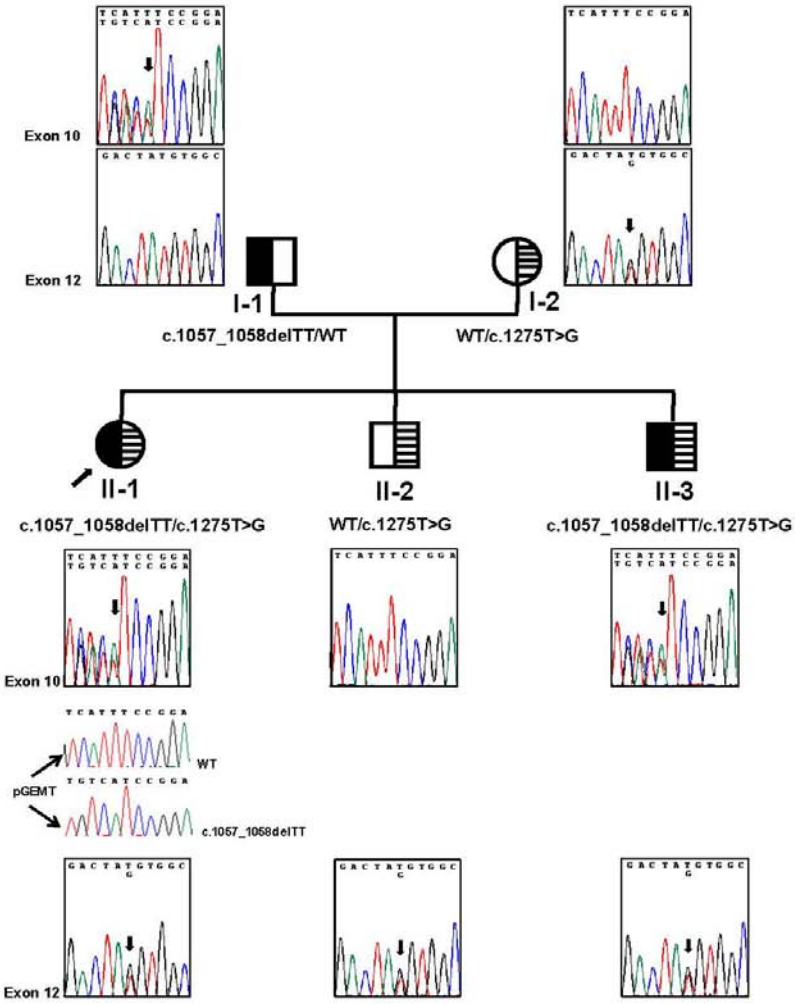

Figure 1: Segregation analysis of the *DUOX2* mutated alleles of the index patient II-1 and the family members. Partial sequencing chromatograms of genomic DNA are shown. The pedigree shows the pattern of inheritance of the mutant *DUOX2* alleles. Squares represent males and circles females. Filled symbols denote affected individuals and half-filled symbols, unaffected heterozygote individuals. The solid symbols indicate the c.1057_1058delTT mutated allele and the hatched symbols the c.1275T>G mutated allele. Sense strand is shown. Note that exon 10 was sequenced with the corresponding intronic reverse primer and the reverse and complementary sequence is shown. Arrows denote the position of identified mutations, single chromatogram peaks indicate homozygosity and two overlapping peaks at the same locus, heterozygosity. The proband is indicated with an arrow. The index patient II-1 and her brother (II-3) were compound heterozygous for c.1057_1058delTT and c.1275T>G. The father was heterozygous for c.1057_1058delTT and the mother, and her healthy brothers (II-2) were heterozygous for c.1275T>G. Partial sequencing chromatograms of wild-type (WT) and mutated (c.1057_1058delTT) alleles corresponding to exon 10 from index patient II-1 cloned into pGEM-T Easy vector are shown.

and manifestations suggesting hypothyroidism.

Written informed consent to participate in the clinical and genetic studies was given by both parents and the research project was approved by the Institutional Review Board.

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

PCR amplification

Genomic DNA was isolated from peripheral blood leucocytes by the standard Cetyltrimetilammonium Bromide (CTAB) method and stored at -20 °C up to the analysis.

The promotor region and all 17 exons of the human *TPO* gene including splicing signals and the flanking regions of each intron, and the complete coding sequence of the human *DUOX2* gene, along with the flanking regions of each intron were amplified using the primers and PCR conditions reported previously [17,48]. The amplified products were analysed in 2% agarose gel.

Single strand conformation polymorphism (SSCP) analysis

SSCP analysis was used to screen for the presence of mutations in each exon of the *TPO* and *DUOX2* genes and their flanking intronic regions. The gel matrix for SSCP contained 8%, 10% or 12% polyacrylamide (29:1) (Invitrogen, Life Technologies), with or without 10% glycerol. Samples were electrophoresed for 17-43 h at a constant temperature (4 °C). DNA was visualized by silver staining.

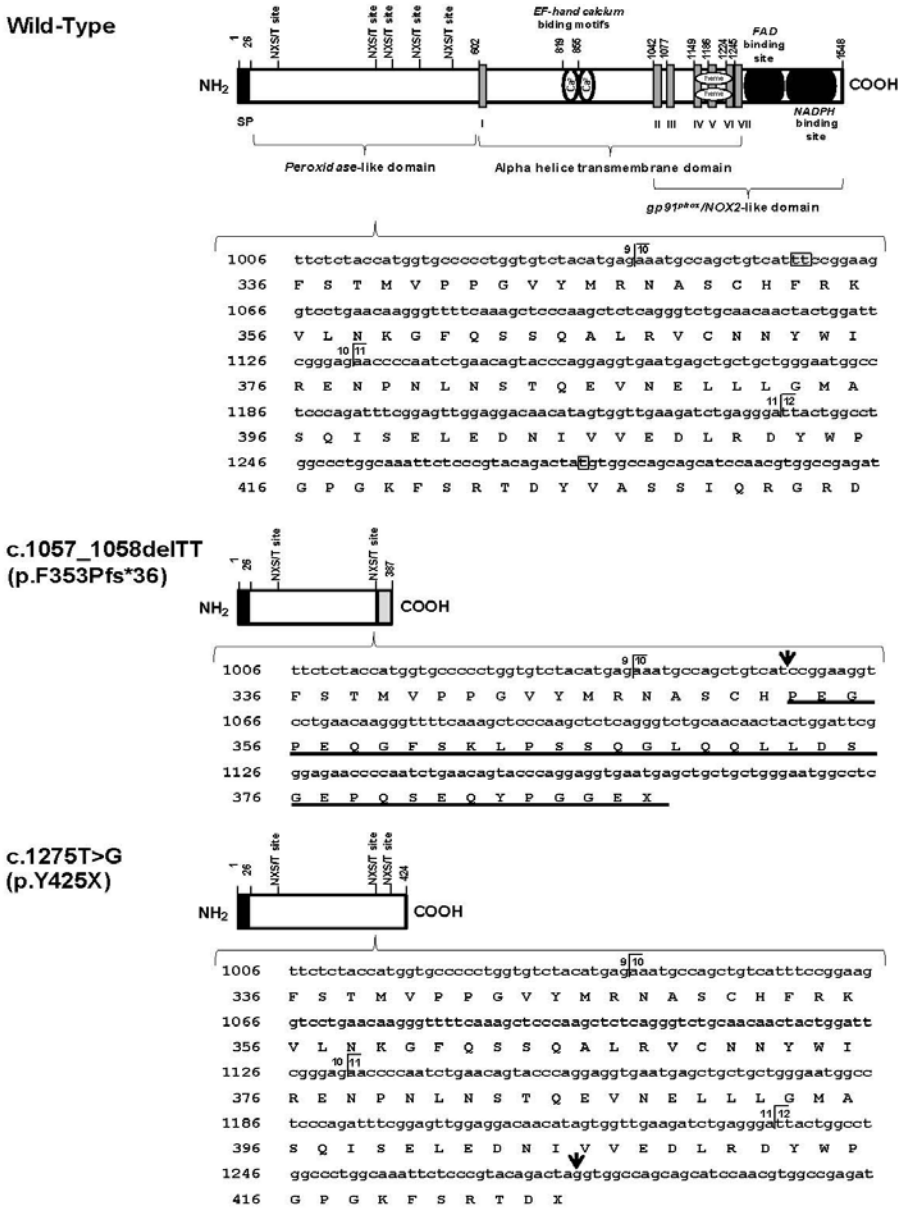

Figure 2: Structural organization of the wild-type and putative mutant DUOX2 proteins**.** The peroxidase-like and gp91^{phox}/NOX2-like domains, glycosilation sites (NXS/T site), EF-hand calcium motifs, and, FAD and NADPH binding sites, drawn to scale, are shown. The seven alpha helice transmembrane domains are represented by boxes. The partial nucleotide and the deduced amino acid sequences from wild-type are reported below of the respective schematic protein diagrams. The nucleotide sequence is given in the upper line, and the amino acid translation (represented by single-letter code) is given below their respective codons. The arrows denote the positions of the c.1057_1058delTT and c.1275T>G mutations and are boxed in the wild-type sequence. The resulting frameshift due to c.1057_1058delTT mutation is underlined. The nucleotide position in human *DUOX* mRNA is designated according to reference sequences (NCBI, accession number: NM_014080.4). The 'A' of the ATG start codon is denoted as nucleotide +1 being the initiator methionine, the codon 1. Indicates exon/exon boundaries and exon numbering is shown.

DNA sequencing

DUOX2 PCR products showing an aberrant pattern in SSCP analysis were purified by GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and directly sequenced using sense- and antisense-specific primers reported previously [17] with the Big Dyedeoxyterminator Cycle Sequencing Kit (Applied Biosystems, Weiterstadt, Germany). The samples were analysed on the 3130xl and 3500xl Genetic Analyzer (Applied Biosystems).

Sequence variants are numbered according to *DUOX2* mRNA reference sequences reported in National Center for Biotechnology Information (NCBI), accession number: NM_014080.4. The DUOX2 gene includes 34 exons, being the first non-coding. The 'A' of the ATG start codon is denoted as nucleotide +1 being the initiator methionine, the codon 1.

Cloning of wild-type and mutated exons 9 PCR fragments

The amplified fragment corresponding to exon 9 from index

patient II-1 was T-A cloned into pGEM-T Easy vector (Promega, Madison, WI, USA). DNA sequencing was performed as described above from wild type and mutant allele clones using the T7 primer.

Results

Promoter and all 17 exons of *TPO* gene and all 33 coding exons of the *DUOX2* gene, along with the flanking intronic sequences, from patient II-1 and healthy controls were screened by SSCP analysis. The analysis of *TPO* PCR products did not show aberrant bands, suggesting the absence of *TPO* gene mutations. Whereas, analysis of *DUOX2* PCR products showed two different patterns of migration that were not detected in the healthy controls. Sequence analysis of the samples showing the abnormal SSCP patterns revealed two mutations. One of them was a novel heterozygous double thymine deletion at nucleotide positions 1057 and 1058 (c.1057_1058delTT) in exon 10 (Figure 1), resulting in a frameshift at amino acid 353 with a putative Premature Termination Codon (PTC) at 36 codons downstream, located in the exon 11 [p.F353Pfs*36] (Figure 2). The c.1057_1058delTT heterozygous state of index patient II-1 was confirmed by cloning in pGEM-T Easy vector and sequencing of both wild type and mutant alleles (Figure 1). The second mutation was a possible previously documented heterozygous thymine-to-guanine transversion at nucleotide position 1275 (c.1275T>G) in exon 12 (Figure 1) which replaces a tyrosine residue at position 425 by a PTC [p.Y425X] (Figure 2). Previously Cortinovis et al. reported the p.Y425X mutation but did not inform what is the nucleotide change that causes the PTC [19]. Two different mutations may generate a stop codon in this position, c.1275T>G (TAT>TAG) or c.1275T>A (TAT>TAA). These findings indicate that index patient II-1 is a compound heterozygous for c.1057_1058delTT/c.1275T>G mutations.

Sequencing analysis of PCR products of exons 10 and 12 from index patient II-1, his father (I-1), his mother (I-2), his unaffected brother (II-2) and his affected brother (II-3) showed that II-2 and II-3 inherited one copy of the c.1057_1058delTT mutation from his father and one copy of the c.1275T>G mutation from his mother (Figure 1). The healthy brother (II-1) is heterozygous for the c.1275T>G mutation and does not carry the c.1057_1058delTT mutation.

Discussion

Here, we present two children from an unrelated family with TCH due to biallelic mutations in the DUOX2 gene. Both affected members had clinical and biochemical criteria suggestive of CH associated with DUOX2 deficiency: intact iodide trapping, IOD, elevated-serum TSH with high levels of serum TG. Molecular analyses indicated that the affected individuals are either compound heterozygous for c.1057_1058delTT/c.1275T>G mutations.

The *DUOX1* and *DUOX2* genes encode similar proteins that are inserted in the apical membrane of thyroid follicular cells. The *DUOX2* gene (GenBank accession number NT_010194) is located on chromosome 15q15.3 spanning 22 Kb of genomic DNA which include 34 exons, being the first non-coding. The mRNA (GenBank accession number NM_014080) is 6,376 nucleotides long and the preprotein is composed of a putative 25 amino acids signal peptide followed by a 1,523 amino acids polypeptide [3]. *DUOX1* gene encodes a homologous protein displaying 83% sequence similarity with DUOX2. The 36 kb *DUOX1* gene consists of 35 exons (being the first two noncoding) and gives rise to a protein of 1551 amino acids whose first 21 correspond to the signal peptide [3]. DUOX2 alterations were first described in 2002 [13]. In the literature, Sanger sequencing has been commonly used for DUOX2 mutations identification in both research and clinical studies. Recently, high throughput sequencing has been reported to be a rapid and effective tool for largest DUOX2 mutation screening for patients with or without family history. The DUOX2 mutations identified in our study and in the literature are reviewed in Table 2. The mutational spectrum shows heterogenous alterations dispersed in the exons and introns of the entire gene. To date, molecular research has shown that 121 pathogenic variations and functional single nucleotide polymorphisms have been found to be associated with TCH, SCH or PCH [8,9,11-13-47]. The most frequent events of deleterious DUOX2 mutations were found to be altered proteins with a single amino acid substitution (78 mutations, see Table 2); followed by deletions/insertion (26 mutations, see Table 2) and nonsense mutations (11 mutations, see Table 2) causing premature truncation of the DUOX2 protein. Only six *DUOX2* splice site mutations have been reported: c.514-2A>G (g.IVS5-2A>G) [12], c.2335-1G>C (g.IVS18-1G>C) [31,36], c.2655-2A>C (g.IVS20-2A>C) [17], c.3516-1G>A (g.IVS26-1G>A) [12], c.3693+1G>T (g.IVS28+1G>T) [12] and c.4240-1G>C (g.IVS31-1G>C) [42]. The p.K530X, p.E897K, p.R1110Q, p.L1160del, p.R1334W and p.L1343F mutations are the most frequently identified DUOX2 mutations in Asian population, whereas the frequent mutation p.S965Sfs*30 was found in Caucasian populations.

The structure of *DUOX2* protein contains three regions: 1) *peroxidase* homology ectodomain (*peroxidase*-like domain): an amino-terminal extracellular region of approximately 600 residues, which has 43% sequence homology with *hemoperoxidasas,* which distinguishes *DUOX* from other family *NOX/DUOX,* 2) a central region encompassing an intracellular loop containing potentially two *EF-hand calcium* binding motifs, which would be involved in regulating the conformation and activity of *NADPH oxidase*; 3) *gp91^{phox}/NOX2*-like domain: a carboxy-terminal region referred to as the comprising the last six transmembrane domains [3-5,48,49] (Figure 2). These six alpha helices include four conserved histidines, considered two coordination sites of prosthetic group of type heme. The C-terminal cytosolic orientation presents a binding site for the FAD and NADPH [3-5,49]. It is believed that this region is responsible for the transfer of electrons from NADPH through the membrane. The c.1057_1058delTT mutation results in a putative truncated protein of 388 amino acids (Figure 2). The functional consequence of the PTC in p.F353Pfs*36 and p.Y425X mutated proteins, could be the complete ability loss to generate thyroid H_2O_2. Both PTCs eliminate the *gp91^{phox}/NOX2*-like domain, and partially the *peroxidase*-like domain (Figures 2). In addition to altered DUOX2 by truncated proteins as a pathophysiological mechanism in the generation of congenital hypothyroidism, the mutated mRNAs generated in our patient could represent adequate targets for Nonsense Mediated mRNA decay (NMD) pathway, a known RNA surveillance mechanism that detects and rapidly degrades selectively mRNAs that contains PTCs and this could also contribute to the development of the disease [50]. Transcripts from genes carrying PTCs are subject to extreme downregulation, often by 10- to 100-fold and sometimes a complete degradation of mutant mRNA occurs. Specifically, when at

Table 2: Spectrum of DUOX2 mutations.

#	Exon/Intron	Nucleotide alteration	Amino acid alteration	References
1	2	c.34delC	p.L12Wfs*5	42
2	3	c.108G>C	p.Q36H	17
3	3	c.127A>T	p.N43Y	30, 42
4	4	c.184C>T	p.P62S	38
5	4	c.214G>T	p.A72S	30
6	4	c.244C>A	p.R82S	11
7	4	c.244C>T	p.R82C	12
8	4	c.287C>T	p.P96L	30
9	4	c.298A>G	p.N100D	9
10	5	c.343G>T	p.D115Y	41
11	5	c.377-379delCCG	p.A126del	12
12	5	c.398T>A	p.I133N	42
13	5	c.413C>T	p.P138L	31, 41
14	5	c.477delC	p.P159Pfs*17	12
15	5	c.512T>C	p.L171P	31
16	Intron 5	c.514-2A>G (g.IVS5-2A>G)		12
17	6	c.518A>G	p.N173S	40
18	6	c.534G>T	p.W178C	12
19	6	c.596delC	p.S199Wfs*122	12, 41
20	6	c.602_603insG skipping of exon 5	p.G201Gfs*100 p.A172Pfs*82	12, 16, 31
21	6	c.605_621delAGCTGGCGTCGGGGCCC	p.Q202Rfs*93	12, 30, 42
22	6	c.616G>T	p.G206V	30
23	6	c.647-656delAGAACCCCCTins15 (NA)	p.Q216Lfs*107	12
24	7	c.804delG	p.R268Sfs*53	41
25	8	c.903G>T	p.W301C	11, 34
26	8	c.908C>G	p.P303R	31, 40
27	9	c.979G>T	p.E327*	12, 26
28	9	c.1021C>T	p.P341S	31
29	10	c.1057_1058delTT	p.F353Pfs*36	Present study
30	10	c.1060C>T	p.R354W	27, 39, 43
31	10	c.1097C>G	p.A366G	42
32	10	c.1126C>T	p.R376W	14, 19, 24
33	11	c.1219G>T	p.V407F	44
34	11	c.1232G>A	p.R411K	42
35	12	NA	p.W414*	19
36	12	c.1253delG	p.G418Afs*65	17
37	12	c.1275T>G	p.Y425*	Present study, 19
38	12	c.1300C>T	p.R434*	13, 29
39	13	c.1424A>G	p.Y475C	19
40	13	c.1435_1440delCTATCCinsAG	p.L479Sfs*3	20, 28, 41
41	13	c.1462G>A	p.G488R	25, 28, 30, 44
42	13	c.1516G>A	p.D506N	16, 19
43	13	c.1537G>A	p.D513N	42
44	13	c.1564delA	p.T522Pfs*64	31

45	14	c.1588A>T	p.K530*	11, 12, 20, 34, 41
46	14	c.1621C>T	p.R541W	42
47	15	c.1709A>T	p.Q570L	27, 31, 43
48	15	c.1736T>C	p.L579P	11, 34
49	16	c.1871delG	p.G624Afs*15	25
50	16	c.1883delA	p.K628Rfs*11	12, 20, 41, 44
51	16	c.1931A>G	p.K644R	38
52	17	c.1946C>A	p.A649E	20, 41, 44
53	17	c.2000delT	p.L667Rfs*9	44
54	17	c.2033A>G	p.H678R	8, 20, 25, 26, 30, 31, 32, 33, 41
55	17	c.2048G>T	p.R683L	11, 34, 37, 38
56	17	c.2056C>T	p.Q686*	13, 27, 43
57	17	c.2101C>T	p.R701*	9, 12, 13
58	17	c.2102G>A	p.R701Q	8, 31
59	17	c.2102_2104delGAG	p.G702del	11
60	17	c.2146delC	p.L716Wfs*34	25
61	18	c.2182G>A	p.A728T	8
62	18	c.2202G>A	p.W734*	9
63	18	c.2203G>A	p.D735N	42
64	18	c.2290C>T	p.R764W	43
65	18	c.2303T>C	p.L768P	12
66	Intron 18	c.2335-1G>C (g.IVS18-1G>C)		31, 36
67	19	c.2335G>A	p.V779M	38, 41, 44
68	19	c.2377_2378dupGA	p.D793Efs*11	25
69	19	c.2524C>T	p.R842*	11, 14, 19, 34
70	20	c.2597T>G	p.M866R	31
71	20	c.2635G>A	p.E879K	11, 12, 20, 30, 34, 41, 44
72	20	c.2654G>A	p.R885Q	20, 30, 41, 44, 45, 47
73	20	c.2654G>T	p.R885L	25, 38, 44
74	Intron 20	c.2655-2A>C (g.IVS20-2A>C)		17
75	21	c.2732C>T	p.S911L	22
76	21	c.2843delG	p.G948Vfs*48	41
77	22	c.2895_2898delGTTC	p.S965Sfs*30	8, 13, 15, 17, 19, 31, 43
78	22	c.2894C>T	p.S965L	38
79	23	c.2944C>G	p.P982A	8, 31
80	24	c.3076C>T	p.Q1026*	15
81	24	c.3082_3085delCTGCinsGCTTCCT	p.L1028Afs*3	43
82	24	c.3096G>C	p.K1032N	41
83	24	c.3116G>A	p.R1039Q	42
84	24	c.3155G>A	p.C1052Y	22, 31
85	24	c.3179C>T	p.A1060V	44
86	25	c.3200C>T	p.S1067L	20, 31, 41
87	25	c.3239T>C	p.I1080T	41, 25, 44
88	25	c.3251G>A	p.R1084Q	11
89	25	c.3264_3267delCAGC	p.A1088Afs*18	36
90	25	c.3329G>A	p.R1110Q	9, 11, 12, 21, 25, 30, 34, 38, 41, 42, 44, 46

91	25	c.3340delC	p.L1114Sfs*56	11, 34
92	25	c.3367G>A	p.A1123T	12, 30
93	25	c.3383G>A	p.R1128H	44
94	25	c.3391G>T	p.A1131S	9, 11
95	25	c.3413C>A	p.A1138D	11, 34
96	26	c.3449A>G	p.Y1150C	8
97	26	c.3478_3480delCTG	p.L1160del	11, 25, 41, 42, 44, 46
98	Intron 26	c.3516-1G>A (g.IVS26-1G>A)		12
99	Intron 26-exon 34	c.3516-?_4647+?del	p.G1172_F1548del	23
100	27	c.3516-3531delGTCCAAGCTTCCCCAG	p.G1172Gfs*14	12
101	27	c.3540T>A	p.Y1180*	42, 46
102	27	c.3541T>G	p.W1181G	9
103	28	c.3616G>A	p.A1206T	9, 39, 44
104	28	c.3632G>A	p.R1211H	33
105	28	c.3652T>C	p.F1218L	12
106	Intron 28	c.3693+1G>T (g.IVS28+1G>T)		12
107	29	c.3799C>T	p.R1267W	9
108	30	c.3967G>A	p.A1323T	11, 35
109	30	c.4000C>T	p.R1334W	11, 12, 30, 34, 46
110	30	c.4027C>T	p.L1343F	11, 34, 35, 37, 38, 41
111	30	c.4040A>G	p.Y1347C	32
112	31	c.4171C>G	p.P1391A	44
113	31	c.4176T>G	p.F1392L	12
114	Intron 31	c.4240-1G>C (g.IVS31-1G>C)		42
115	32	c.4334T>A	p.V1445E	44
116	32	c.4348T>C	p.Y1450H	44
117	33	c.4405G>A	p.E1469K	44
118	33	c.4408C>T	p.R1470W	38
119	33	c.4475G>A	p.R1492H	11, 41
120	34	c.4552G>A	p.G1518S	23
121	34	c.4637A>G	p.E1546G	31

Sequence variants are numbered according to DUOX2 mRNA reference sequences reported in National Center for Biotechnology Information (NCBI), accession number:NM_014080.4. The DUOX2 gene includes 34 exons, being the first non-coding. The 'A' of the ATG start codon is denoted as nucleotide +1 being the initiator methionine, the codon 1. Splicing mutations are annotated by using cDNA sequences and old nomenclature (g.IVS) is included. Frameshifting mutations are designated by "fs" and stop codón with "*", the length of the shifted open reading frame include the first affected amino acid. NA: Not Available.

least one intron downstream from the PTC and a minimal distance of 50 to 55 nucleotides between the PTC and the final intron, the mRNA is considered defective and is subjected to NMD pathway. The distance between the PTC in p.F353Pfs*36 mutated protein and the start of intron 11 is of 69 nucleotides, whereas the distance between PTC in p.Y425X mutated protein and the start of intron 12 is of 123 nucleotides. Therefore, the position of both PTCs in our study satisfies the condition in order to NMD pathway can be activated.

CH is the most frequent endocrine disease in the infant and one of the most common preventable causes of cognitive and motor impairment. The incidence of CH has increased in recent years with a current prevalence of 1:1400 to 1:2800 [51]. This increase is mainly due to the widespread lowering of TSH screening cutoffs, which has led to detecting mildest forms of CH with normally positioned thyroid gland [52,53]. Whereas the majority of the cases of CH are due to dysembryogenesis or dysgenesis of the thyroid gland (agenesis, ectopia, hypoplasia), one fifth of these hypothyroid newborns have a defect in genes involved in thyroid hormone biosynthesis (dyshormonogenesis) [1]. The prevalence of DUOX2 mutations in thyroid dyshormonogenesis is estimated to be in the range of 29-45 % [8,13,25,31]. In Asian, the prevalence of DUOX2 mutations in thyroid dyshormonogenesis is higher than in Caucasians [38]. Recent studies were carried out from Chinese patients with CH in order to establish a genotype-phenotype correlation [11,12,34,37]. The genotype-phenotype analysis of 131 Chinese patients with CH is summarized in Table 3. So, one or two DUOX2 mutations were associated with SCH or TCH, whereas patients with three or more DUOX2 pathogenic variants were mostly associated with PCH (Table

Table 3: Genotype-phenotype correlation in 131 Chinese patients with congenital hypothyroidism with DUOX2 variants [11,12,34,37].

# of DUOX2 mutations	# of patients	TCH	PCH or APCH	SCH or MPCH	ND
1	76	51 (67%)	7 (9%)	15 (20%)	3 (4%)
2	37	27 (73%)	2 (5,4%)	3 (8.1%)	5 (13.5%)
3	16	2 (12.5%)	14 (87.5%)	0	0
4	1	0	1 (100%)	0	0
5	1	0	1 (100%)	0	0
Total	131	80 (61.1%)	25 (19.1%)	18 (13.7%)	8 (6.1%)

TCH: Transient Congenital Hypothyroidism; PCH: Permanent Congenital Hypothyroidism; APCH: Apparent Permanent Congenital Hypothyroidism; SCH: Subclinical Congenital Hypothyroidism; MPCH: Mild Permanent Congenital Hypothyroidism; NA: Not Available.

3). In addition, compound DUOX2 mutations with other thyroid genes have been identified: TSHR [30,35,38,44], DUOXA2 [45,47], TPO [37,38,42,46], TG [11,38,43] and SLC26A4 [38] genes. However, to date are no functional evidence that the addictive effect of digenic mutations contribute to the CH phenotype.

In conclusion, the present study clearly established that total loss of DUOX2 activity by PTC causes TCH phenotype. Our findings also provide further evidence that the transient or persistent variability of the CH phenotype is not directly related to the number of mutant *DUOX2* alleles, suggesting the existence of ethnic differences, additional genetic and epigenetic changes, environmental/ geographical factors and the existence of additional H2O2 supply provided by DUOX1, which play a role in the determining severity of this type of form of thyroid dyshormonogenesis.

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