



# Genotype and phenotype correlation in a cohort of Chinese congenital hypothyroidism patients with DUOX2 mutations

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**Background:** This study aimed to explore the relationship between the phenotype and genotype of congenital hypothyroidism (CH) caused by dual oxidase 2 (DUOX2) mutation in Chinese children, and to investigate the genetic causes of permanent and transient hypothyroidism through next-generation genetic testing technology and long-term clinical follow-up data.

**Methods:** We recruited 61 patients with thyroid stimulating hormone (TSH) levels of >10 mIU/mL during newborn screening, clinical diagnosis of CH, and L-thyroxine (L-T4) oral treatment within 1 month of birth; they were followed up until the present. All CH infants and their parents were genotyped using whole-exome sequencing (WES); DUOX2 variants were detected in 20 infants, and the longitudinal prognosis, genotype, and phenotype correlations were analyzed.

**Results:** Biallelic DUOX2 mutations were detected in 20 participants. All of them were born full term. All patients were treated with L-T4 when diagnosed with CH; 9 of them stopped L-T4 eventually before 3 years old; and 2 were treated with a reduced dose of L-T4 (12.5 µg per day). The others were still treated with L-T4 at a dose of 37.5–87.5 µg per day. Of these 20 participants, 5 carried an R1110Q variant and 5 carried K530X variants. A total of 7 novel variants were discovered in our cohort. The variants carried in transient CH patients were located extracellularly and not near the functional domain.

**Conclusions:** Most CH patients with DUOX2 mutations were those with transient or subclinical CH. The R1110Q, R885L, and K530X were the most common variants in our Chinese cohort. The R1110Q and K530X variants may play a founder effect in the transient CH. The R885L variant may play a benign role in transient CH. Intracellular variants or those near the functional domain may cause permanent CH.

**Keywords:** Congenital hypothyroidism (CH); dual oxidase 2 (DUOX2); Chinese; phenotype genotype

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## Introduction

Early in the 1980s, the thyroid H<sub>2</sub>O<sub>2</sub> generator was found in the apical plasma membrane of pig open follicles (1). In 1999, Leseney *et al.* found biochemical characterization of a Ca<sup>2+</sup>/NAD(P)H-dependent H<sub>2</sub>O<sub>2</sub> generator in human thyroid tissue (2). The dual oxidase 2 (DUOX2) gene was firstly purified in 1999 and located on chromosome 15q21.1, consisting of 34 exons (3). In 2007, the mouse

DUOX2 mutation was reported to have occurred spontaneously at the Jackson Laboratory and was named “thyroid dysmorphogenesis” to signify the characteristic defect of thyroid hormone synthesis (4). This is currently the only known gene that causes transient congenital hypothyroidism (CH), but its mechanism has not yet been clarified. The protein encoded by this gene is a glycoprotein and a member of the nicotinamide adenine dinucleotide

phosphate (NADPH) oxidase family. The synthesis of thyroid hormone is catalyzed by a protein complex located at the apical membrane of thyroid follicular cells. This complex contains an iodide transporter, thyroperoxidase, and a peroxide generating system that includes this encoded protein and dual oxidase 1 (DUOX1) (5).

In 2002, Moreno *et al.* found the inactivated mutation of DUOX2 gene in 9 CH patients (1 permanent and 8 transient) (6). After that, additional cases were reported with DUOX2 gene mutations (7), and they found that a missense mutation (p.R1110Q) may cause thyroid goiter. In China, Liu *et al.* found 2 novel missense mutations (p.R354W and p.A1206T) impaired H<sub>2</sub>O<sub>2</sub> production and caused CH and goiter (8).

However, none of the above studies have clarified the pathogenesis of transient hypothyroidism, and the potential mechanism may not be discovered. Long-term outcome data are insufficient to determine further outcomes of transient CH. Therefore, we have endeavored to spend years following up these patients and intend to explore the association between the DUOX2 genotype and phenotype in the treatment of patients with DUOX2 mutation hypothyroidism. We present the following article in accordance with the MDAR checklist (available at <http://dx.doi.org/10.21037/atm-20-7165>).

## Methods

### Participants

A total of 61 patients who presented with low thyroxine (T4) and free thyroxine (fT4) levels, and thyroid stimulating hormone (TSH) levels >10 mIU/mL detected by the national neonatal newborn screening program were recruited (9). Whole-exome sequencing was performed and 20 patients (7 males, 13 females) among them were found to harbor biallelic DUOX2 mutations. All patients were double checked in our hospital and thereafter treated with L-thyroxine (L-T4) at an initial dosage of 10–15 µg/kg per day (9). The L-T4 dosages were titrated at the suggested serum level of TSH <3 mIU/mL (9). Transient CH (TCH) or permanent CH (PCH) classification of the patients was dependent on the need of L-T4 in maintaining euthyroidism. After withdrawal of therapy, serum TSH, fT3, and fT4 of all patients were followed once or twice a year until the present.

This study was approved by the ethics committee of Children's Hospital of Fudan University (No. 2015-183).

All participants consented to this research, as provided by their parents or guardians. The samples were collected according to the World Medical Association Declaration of Helsinki (as revised in 2013).

### Study methods

#### Statistics Analysis

Continuous variables are expressed in mean ± standard deviation (SD). Comparison between permanent and transient hypothyroidism was performed by unpaired t-test. P values <0.05 were considered statistically significant. The statistical analysis was performed using R (v. 4.0.3, [www.r-project.org](http://www.r-project.org))

#### Whole-exome sequencing

Frozen peripheral blood samples were sent to a service provider for whole-exome sequencing (WES) and disease related mutation analysis (Shanghai Gempile Biotechnology Co., Ltd., Shanghai, China). Total DNA was isolated using the UnigeneDx Blood DNA extraction Kit (Unigene Laboratories Inc., Boonton, NJ, USA), and DNA concentration was determined using the Qubit dsDNA HS assay kit (Life Technologies, Carlsbad, CA, USA). Exome sequencing libraries were prepared using the KAPA Hyper Prep Kit (KAPA Biosystems, Roche, Indianapolis, IN, USA), and enrichment was performed with the SeqCapEZ MedExome Kit (NimbleGen, Roche, Indianapolis, IN, USA) following the manufacturer's instructions. Briefly, genomic DNA was sheared to an average size of 180–300 bp using the Covaris M200 sonicator (Covaris, Inc., Woburn, MA, USA). About 300 ng fragmented genomic DNA was used for library construction. The DNA libraries were then pooled as 4-plex or 6-plex for capturing using the SeqCapEZ MedExome probes. Libraries were sequenced with the Illumina HiSeq platform using 150 bp paired-end sequencing.

#### Bioinformatic analysis

An average of 10 Gb raw data (Fastq) as input was generated for each sample by Illumina sequencers. Firstly, the paired-end reads were performed for quality control using FastQC version 0.11.5 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc>). Secondly, Burrows-Wheeler Aligner (BWA) version 0.7.15 (10) was used to align sequencing reads to the reference genome GRCh38. Sequence alignment/map (SAM) format files were generated by BWA. Thirdly,

**Table 1** Genes which are reported related with congenital hypothyroidism

Genes	Location	Exons	Full name
<i>SLC26A4 (PDS)</i>	7q31	23	Solute carrier family 26 (anion exchanger), member 4
<i>TSHR</i>	14q31	12	Thyroid stimulating hormone receptor
<i>TPO</i>	2p25	19	Thyroid peroxidase
<i>MCT8(SLC16A2)</i>	Xq13	6	Solute carrier family 16 (monocarboxylic acid transporters), member 2
<i>TG</i>	8q24	52	Thyroglobulin
<i>PAX8</i>	2q13	12	Paired box (PAX) family of transcription factors
<i>TSHB</i>	1p13	3	Thyroid stimulating hormone, beta
<i>THRB</i>	3p24.2	17	Thyroid hormone receptor, beta
<i>DUOX2</i>	15q15.3	34	Dual oxidase 2
<i>SLC5A5 (NIS)</i>	19p13.11	16	Solute carrier family 5 (sodium/iodide cotransporter), member 5
<i>NKX2-1</i>	14q13	3	NK2 homeobox 1
<i>FOXE1</i>	9q22	1	Forkhead box E1
<i>PDE8B</i>	5q13.3	26	Phosphodiesterase 8B
<i>GNAS</i>	20q13.3	16	GNAS complex locus
<i>TTF2</i>	1p22	25	Transcription termination factor, RNA polymerase
<i>POU1F1</i>	3p11	6	POU class 1 homeobox 1
<i>GLIS3</i>	9p24.2	19	GLIS family zinc finger 3
<i>DEHAL1 (IYD)</i>	6q25.1	7	Iodotyrosine deiodinase
<i>THRA</i>	17q11.2	11	Thyroid hormone receptor, alpha
<i>IGSF1</i>	Xq25	23	Immunoglobulin superfamily, member 1
<i>DUOXA2</i>	15q15.1	6	Dual oxidase maturation factor 2

the SAM format files were further processed to binary SAM (BAM) files using Samtools version 1.3.1 (11), then duplicates were removed using Picard version 2.5 (12). After these processes, variant calling was performed by GATK version 3.5 (13), and the Vcf file was generated. Finally, we used Annovar software to annotate the variants from the Variant Call Format (VCF) file and integrate information from multiple databases. The final variants could feed to the downstream advanced analysis pipeline.

### Variation filtering and validation

Initially, we focused on variants in 30 candidate genes which have been implicated in CH or thyroid dysgenesis. This core gene list was determined by databases such as Online Mendelian Inheritance in Man (OMIM) and GeneReviews, as well as published literature, and is supplied in *Table 1*. Variants

were analyzed following the basic criteria from American College of Medical Genetics (ACMG) guideline (14). Confirmation of variants identified by WES was carried out using standard Sanger sequencing using polymerase chain reaction. Polyphen-2 (<http://genetics.bwh.harvard.edu/pph2/>) and mutation taster (<http://asia.ensembl.org/index.html>) tools were used for all novel variants to predict whether they were pathogenic mutations.

### Results

In these 61 patients, we all carried out genetic test. Twenty of them were with DUOX2 variations. Six of them were TSHR variations, 2 of them were GNAS variations, 2 of them were DUOXA2 variations, 1 of them were NIS variation, 1 of them was TG variation, the others were negative.

### *Thyroxine level of participants with mutations in DUOX2*

T4 level was  $3.03 \pm 1.49$   $\mu\text{g/dL}$  in PCH group versus  $4.73 \pm 2.33$   $\mu\text{g/dL}$  in TCH group. FT4 level was  $0.53 \pm 0.18$   $\text{ng/dL}$  in PCH group versus  $0.67 \pm 0.27$   $\text{ng/dL}$  in TCH group. TSH level was  $73.63 \pm 18.72$   $\text{mIU/mL}$  in PCH group versus  $53.41 \pm 32.02$   $\text{mIU/mL}$  in TCH group. No significant difference was found in each above groups.

### *Clinical course of participants with mutations in DUOX2*

In most participants, thyroid function was improved and the dose of L-T4 could be reduced by around 2–4 years of age, except for the siblings in cases 13 and 14. Some 10 of the 20 patients aged 3–7 years old were able to have their L-T4 dose gradually reduced and then eventually stopped altogether at the age of 2–3 years. After stopping L-T4, these patients still followed up in our clinic and the levels of thyroid hormone continued to be normal. Case 13 and 14 were siblings with same homozygous mutations of p.R1110Q, and both developed PCH. They were born in 2010 and 2013, respectively; their doses of L-T4 were 50  $\mu\text{g}$  per day at birth and are now 87.5  $\mu\text{g}$  per day to maintain normal levels of TSH, fT3, and fT4 and PCH. Case 9 was lost due to ceased contact. In the other participants, 2 (cases 18 and 19) have had their L-T4 doses reduced to 12.5  $\mu\text{g}$  and might try to stop L-T4 in the future follow-up. The participant known as case 4 has had their dose reduced to 25  $\mu\text{g}$  per day. Cases 1 and 7 have reduced theirs to 37.5  $\mu\text{g}$  per day. Case 10 and 20 (n=2) are maintained 50  $\mu\text{g}$  per day. All patients were assessed as physically and psychologically normal until recently. This phenomenon indicates that most patients with DUOX2 mutations have slightly subclinical hypothyroidism (Table 2). During puberty, none of the transient cases showed signs of hypothyroidism (elevation of TSH or reduction of fT4 level).

### *Genotypes of patients with mutations in DUOX2*

All participants had biallelic DUOX2 variants (Table 2). All parents of the participants in this study are carriers of monoallelic DUOX2 mutations, and they did not have a history of hypothyroidism. Of these 20 patients, 5 carried an R1110Q variant and 5 carried K530X variants, which indicated that both variants might be the hot sites of DUOX2 in our Chinese CH cohort. A total of 7 novel variants were detected (Table 2). Prediction of the functional effects of the novel missense variants

was made using polyphen-2 and mutation taster. Novel variants c.3363\_3364delCA (p.D1121fsX48), c.C3956T (p.T1319I), c.C411A (p.D137E), c.T959C (p.L320P), c.3516\_3531delGTCCAAGCTTCCCCAG, and c.3285\_3286delTT were predicted to be disease causing by mutation taster and probably damaging by polyphen-2. Novel variant c.G1127T (p.R376L) was predicted as disease causing by mutation taster but benign by polyphen-2.

## Discussion

As reported, the incidence of CH in China is about 1:2,050 (15), and the global incidence rate is about 1:581–4,400 (16–19). Most of the CH is sporadic, but it is also related to genetic factors. Currently known mutations include NKX2-1, NKX2-5, PAX8, TTF2, and thyrotropin receptor (TSHR) (20). Thyroid hormone abnormalities are associated with genetic defects involved in thyroid hormone synthesis. Pathogenic genes include DUOX2, thyroid peroxidase (TPO), solute carrier family 5 or sodium iodide symporter (SLC5A/NIS), thyroglobulin (TG), iodine tyrosine deiodinase (IYD), dual oxidase maturation factor 2 (DUOXA2), and solute carrier family 26 member 4 (SLC26A4) (20).

Reports on CH caused by the DUOX2 gene have been gradually increasing. Since 2014, after the application of next-generation sequencing (NGS) technology to clinical genetic diagnosis services, large-scale research has become more common. Increasing numbers of mutation sites have been discovered. We reviewed the majority of published research and listed the reported mutation sites correlated with the natural course of CH (5,6,21–34) (Table 3). All these papers analyzed the correlation between phenotype and genotype, as it is considered that either TCH or PCH might be determined by the different mutations. Our study found that patients with homozygous variants of R1110Q may have PCH, and heterozygous variations may lead to TCH, consistent with some previous findings. In the results of other research institutions in China, the K530X and R885L variants were found to be common in the Chinese population (22,31,35), and these findings concur with our findings. In the majority of our cases, patients with R885L variants only had TCH, which indicated the R885L might be a benign variant. We also found 8 participants (case 2, 3, 10, 12, 16, 17, 18, and 20) with 7 novel variant sites that were predicted by software to be pathogenic. Most of these participants were TCH while cases 10, 16, and 20 were PCH. Cases 16 and 20 carried the same variant of A1206T,

**Table 2** Phenotypes and genotypes data of our cohort

Case	Sex	Age	T4 (µg/dL) (4.5–15.4)	TSH (µIU/mL) (0.25–7.31)	FT4 (ng/dL) (0.5–2.3)	Allele 1, Amino acid	Allele 2, Amino acid	Current treatment	Clinical course
								Oral treatment of L-T4	
1	F	5	4.33	37.5	0.77	p.K530X	p.R1211H	37.5 µg per day	Permanent
2	M	5	4.74	28.9	0.62	p.R1110Q	p.D137E	Stopped	Transient
3	F	5	2.65	>100	0.38	p.D1121fsX48	p.R1110Q	Stopped	Transient
4	F	12	3.45	79.2	0.67	p.R683L	p.L1343F	25 µg per day	Permanent
5	M	7	9.32	13.01	1.06	p.R885L	p.R885L	Stopped	Transient
6	M	6	4.84	36.72	0.54	p.K530X	p.S199fs	Stopped	Transient
7	F	10	2.43	53.4	0.43	p.R683L	p.R885Q	37.5 µg per day	Permanent
8	F	4	7.87	72.51	0.98	p.L1343F	p.R683L	Stopped	Transient
9	F	7	0.58	100	0.13	p.R683L	p.L1343F	No data	No data
10	M	7	0.96	100	0.19	p.R885L	p.L320P	50 µg per day	Permanent
11	M	8	3.94	100	0.49	p.K530X	p.K530X	Stopped	Transient
12	F	4	5.47	27.83	0.62	p.R885L	p.Q570X	Stopped	Transient
13	M	10	1.33	78.2	0.53	p.R1110Q	p.R1110Q	87.5 µg per day	Permanent
14	F	7	1.45	89.3	0.34	p.R1110Q	p.R1110Q	87.5 µg per day	Permanent
15	F	4	1.98	100	0.53	p.R885L	p.K530X	Stopped	Transient
16	F	6	3.62	38.3	1.09	p.A1206T	p.R376L	Stopped	Transient
17	M	5	2.87	63.4	0.42	p.T1319I	p.K530X	Stopped	Transient
18	F	2	4.44	81.65	0.65	p.R1110Q	p.Gly1173SerfsX14	12.5 µg per day	Mild permanent
19	F	5	4.53	75.2	0.53	p.K530X	p.Tyr1096SerfsX12	12.5 µg per day	Mild permanent
20	F	6	4.33	68.2	0.67	p.L1343F	p.A1206T	50 µg per day	Permanent

No data: patients lost to follow-up.

which hinted that the A1206T variant might be the cause of PCH.

Through the changes in amino acids at these sites, as well as systematic reviews of other literature, we have some hypotheses for the pathogenesis of PCH and TCH. The possible mechanism by which DUOX2 mutations cause transient CH is as follows. A possible reason was raised in 2016 by Maruo *et al.*, who investigated the prognosis of patients with CH due to DUOX2 mutations and raised the hypothesis that DUOX1 compensated the DUOX2 mutation in thyroid cells which produce H<sub>2</sub>O<sub>2</sub> for tissue and coupling of tyrosine in the infancy and adult period. The expression level of DUOX1 is one-fifth of DUOX2. Even though DUOX2 enzyme activity is lost, DUOX1 maintains low levels of H<sub>2</sub>O<sub>2</sub> throughout life. In the neonatal period, the demand for thyroid hormone

(10–15 mcg/kg) is 5–7 times higher than that of adults (2 mcg/kg), then, the demand for thyroid hormones in infancy is gradually reduced. In the neonatal and infancy periods, the supply of H<sub>2</sub>O<sub>2</sub> using DUOX1 alone may be insufficient, so individuals with DUOX2 deficiency are prone to signs of CH. As thyroid hormone requirements decrease during development, DUOX1 produces sufficient H<sub>2</sub>O<sub>2</sub> to maintain thyroid hormone synthesis, regardless of DUOX2 mutations (36). This hypothesis may explain the occurrence of TCH, but it cannot explain the phenomenon of PCH.

In our study, variants of PCH patients were almost near the functional domain of DUOX2 (*Figure 1*), which may affect the normal expression of the gene. This might be a possible mechanism of PCH. Stop codon mutations are considered pathogenic in ACMG guidelines. However,

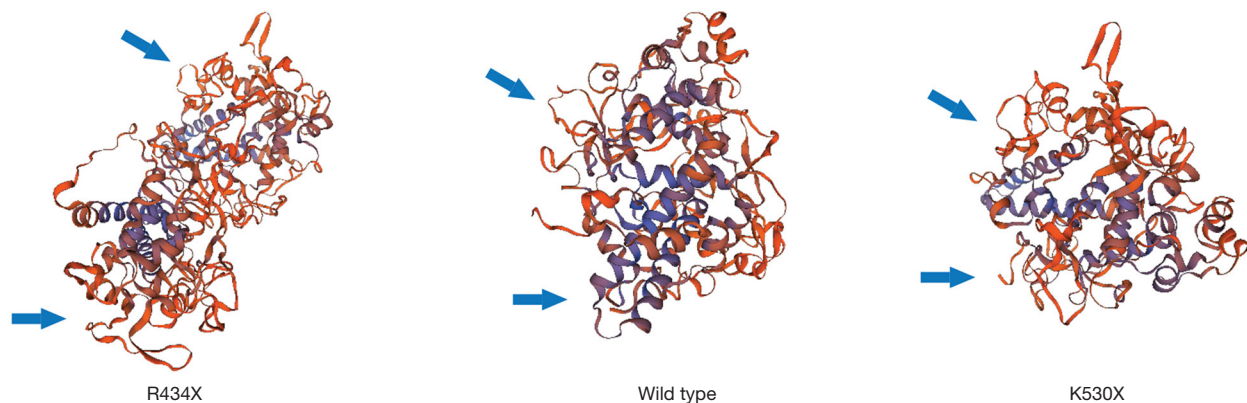
**Table 3** Reported natural course of congenital hypothyroidism in previous researches

Year	First author	Variants				Clinical course
		Allele 1	Amino acid	Allele 2	Amino acid	
2002	Jose C	c.1300C>T	R434X	c.C1300T	R434X	Permanent
		c.2056C>T	Q686X	Wild type	Wild type	Transient
		c.2101C>T	R701X	Wild type	Wild type	Transient
		c.2895-2898del	S965fsX994	Wild type	Wild type	Transient
2005	Maria Cristina Vigone	c.2524C>T	Arg842X	c.1126C>T	p.Arg376Trp	Permanent
2006	Viviana Varela	c.108G>C	R86H	c.1253delG	p.G418fsX482	Unknown
2007	Helmut Grasberger		Q36H		R376W	Complete
			D506N			Partial
2008	Maruo, Yoshihiro	c.1435_1440delCTATCCinsAG	p.L479SfsX2	c.1883delA	p.K628RfsX10	Transient
		c.1588A>T	p.K530X	c.2635G>A; c.3200T>C	p.E876K; p.L1067S	Transient
		c.2033A>G	p.H678R	c.3200T>C	p.L1067S	Transient
		c.1946C→A	p.A649E	c.2654G>A	p.R885Q	Transient
2008	Ohye, Hidemi		p.R1110Q		p.R1110Q	Permanent goiter
2009	Tonacchera, Massimo		p.S911L		p.C1052Y	Permanent
2010	Hoste, Candice	c.4552G>A	p.Gly1518Ser	Wild type	Wild type	Transient
2014	Cangul, Hakan	c.1300C>T	p.R434X	c.1300C>T	p.R434X	Permanent
2014	Jin, Hye Young	c.1462G>A	p.G488R	c.1462G>A	p.G488R	Transient
2015	Fu, C		p.L1114SfsX56		p.K530X	Transient
			p.L1114SfsX56; W301C		p.K530X	Permanent
2016	Park, Kyoung-Jin	c.4334T>A	p.V1445E	c.3329G>A	p.R1110Q	Permanent
		c.3616G>A	p.A1206T	c.3478delCTG	p.L1160del	Permanent
		c.4348T>C	p.Y1450H	c.1462G>A	p.G488R	Permanent
		c.4171C>G	p.P1391A	c.1946C>A	p.A649E	Permanent
		c.1462G>A	p.G488R	Wild type	Wild type	Permanent
		c.2335G>A	p.V779M	c.1462G>A	p.G488R	Permanent
		c.1462G>A	p.G488R	Wild type	Wild type	Permanent
		c.3239T>C	p.I1080T	Wild type	Wild type	Not reported
	p.G488R	Wild type	Wild type	Not reported		
2016	Fu, C		p.K530X		p.K530X	Transient
			p.L1114SfsX56		p.K530X	Transient

**Table 3** (continued)

Table 3 (continued)

Year	First author	Variants				Clinical course	
		Allele 1	Amino acid	Allele 2	Amino acid		
2016	Tan		p.K530X		p.K530X	Transient	
			p.K530X		p.K530X	Transient	
			p.K530X		p.K530X	Transient	
			p.K530X		p.K530X	Mild permanent	
			p.K530X		p.Q202RfsX93	Transient	
		c.647-656del10ins15		p.K530X		p.K530X	Mild permanent/ transient
			p.R701X		p.K530X	Mild permanent/ transient	
2016	Yoshihiro Maruo		p.L1343F		p.R885Q	Mild permanent	
		c.2033A>G	p.H678R	rs145061993	p.V779M	Permanent	
2017	Aycan, Zehra	c.1300C>T	p.R434X	c.1300C>T	p.R434X	Permanent	
2018	Liu		p.R354W			Transient	
			p.A1206T			Transient	

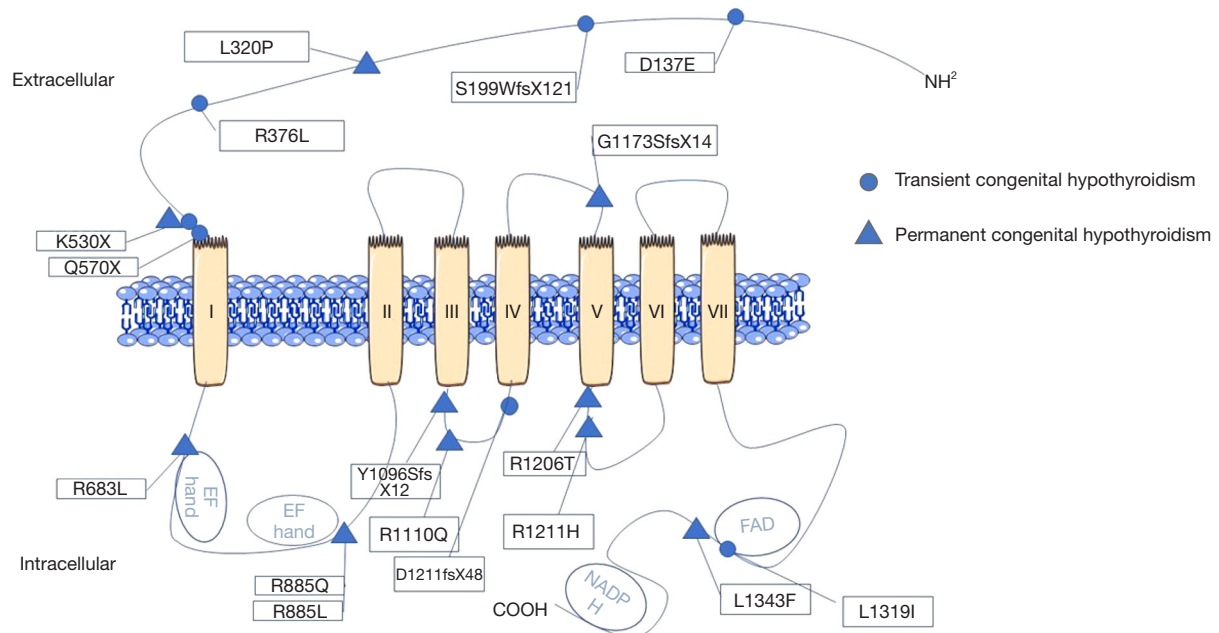


**Figure 1** Predicted structures of different *DUOX2* gene variants. The structure of the *DUOX2* indicated by the arrow in the figure is different. The structure of R434X is relatively loose and partially missing. The protein structure change caused by K530X is mild. *DUOX2*, dual oxidase 2.

most patients with a K530X variant were TCH while most patients with an R434X variant were PCH. We used software (<https://www.swissmodel.expasy.org/interactive>) to predict protein structure, and found the predicted protein structure of K530X was more similar and relatively intact compared with the wild type, but the predicted protein

structure of R434X was more variable (*Figure 2*). The difference of protein structure could alter the activity of the *DUOX2* protein, which may be the cause of differences in their natural course of disease.

This study suggests that our patients with congenital hypothyroidism caused by mutations in the *DUOX2* gene



**Figure 2** All variants detected in our cohort and their position in the functional domain of *DUOX2* gene. *DUOX2*, dual oxidase 2.

are partially transient hypothyroidism. The important findings can be translated into reference materials for clinicians diagnosis and treatment behaviors. And there is a big expectation that the patients don't need to take thyroxine for life, so that disease burden is decreased, and gain increasing compliance.

However, there are still limitations in our study. Thyroid globulin levels were not measured in our cohort. We were also unable to verify the functions of novel variants. Therefore, functional tests of novel *DUOX2* variants such as constructing animal models or iPSC cells should be carried out in the future.

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## Footnote

**Reporting Checklist:** The authors have completed the MDAR checklist. Available at <http://dx.doi.org/10.21037/atm-20-7165>

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**Conflicts of Interest:** All authors have completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/atm-20-7165>). The authors have no conflicts of interest to declare.

**Ethical Statement:** The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by ethics board of Children's Hospital of Fudan University (No. 2015-183 the registration number of ethics board) and informed consent was taken from all individual participants.

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