Mutation Screening of DUOX2 Gene in Children with Congenital Hypothyroidism

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Abstract

Background: Congenital hypothyroidism(CH) is generally known as the most common neonatal endocrine disorder. However, the mutational spectrum of DUOX2 gene and the relationship between genotype and phenotype have not been fully established among Chinese CH patients. Therefore, The aim of this study was to screen DUOX2 mutations in Chinese patients with CH and to research the relationship between DUOX2 genotype and clinical phenotype.

Methods: Eighty-six patients with CH were recruited from northeastern region of China. Peripheral venous blood samples were collected, genomic DNA was extracted , PCR and next-generation sequencing (NGS) were used to analyze all exons of DUOX2. Detailed medical data were collected, and the relationship between DUOX2 genotype and the clinical phenotype was preliminarily investigated.

Results: NGS analysis of DUOX2 gene identified a total of 20 different gene variants in 26 patients(30.2%), which was consistent with the gene variants in Asian populations, among these variants 16 were known to be pathogenic or likely to be pathogenic, and four were suspected to be of uncertain significance. Three mutations (p.K530X, p.L1343F and p.R1110Q) were highly recurrent in our patient cohort. By using protein homology modeling method, the analysis of its three-dimensional structure suggested that the mutations p.336_337del and p.T1107fs caused the change of the protein.

Conclusions: In our study, p.336_337del and p.T1107fs were found to be novel variants and p.K530X with the highest prevalence. Children with DUOX2 single allele heterozygous mutation or compound heterozygous mutation exhibited different morphological developments of the thyroid.

Background

Congenital hypothyroidism(CH) is generally known as the most common neonatal endocrine disorder and occurs approximately with a prevalence of one in 2000 to 4000 infants[1]. Genetic and environmental factors are reported to be associated with CH[2]. Based on genetic alterations, CH can be classified into two groups: of which thyroid gland dysgenesis accounts for 80%~90%, caused by disorders of thyroid gland development including thyroid dysgenesis, comprising agenesis, ectopy, or hypoplasia. It has been linked to gene mutations in the thyroid-stimulating hormone receptor (TSHR), the paired box gene 8 (PAX8), the thyroid transcription factors 1 (TTF-1/NKX2.1) gene, the thyroid transcription factors 2 (TTF-2/FOXE1) gene and the NK2 transcription factor related locus 5 (NKX2-5) gene[3]. Thyroid gland dyshormomogenesis accounts for about 10%~15%, mainly caused by deficiencies in the organification of iodide and is considered as associated with mutations in the thyroglobulin (TG) gene, the thyroperoxidase (TPO) gene, the sodium iodide symporter (NIS) gene, the dual oxidase2 (DUOX2) gene and the dual oxidase maturation factor 2 (DUOXA2) gene[4].

Although most cases of CH patients are sporadic, studies have shown that defects in iodide organification have been confirmed as autosomal recessive genetic model[5,6]. Besides, defects of human

DUOX2 gene are reported to be one of the important causes of CH due to thyroid gland dyshormomogenesis. DUOX2 is located on chromosome 15 and consists of 34 exons. DUOX2 is a protein composed of 1548 amino acids with a 26 amino-acid signal peptide located at the apical membrane of thyrocytes playing an essential role of hydrogen peroxide generation[7]. Up to date, the mutational spectrum of DUOX2 gene and the relationship between genotype and phenotype have not been fully established yet, and little is known about its prevalence among Chinese CH patients. However, it is known that both biallelic and monoallelic DUOX2 gene mutations may lead to a wide spectrum of clinical phenotypes ranging from mild subclinical congenital hypothyroidism (SCH) to permanent congenital hypothyroidism (PCH).

The aim of this study is to establish the prevalence of mutations in DUOX2 gene and explore the relationships between DUOX2 genotypes and clinical phenotypes among among patients with CH in North-East of China.

Methods

Study population

A total of 86 patients (40 males, 46 females; average age: 7±3 years) were recruited from Shengjing hospital of China Medical University based on routine newborn screening result. Newborn screening was done with filter paper for CH between 72h and seven days after birth. Blood samples were collected from the heel and TSH level was measured using an enzyme linked immunosorbent assay. Patients with TSH levels≥10μIU/mL during newborn screening were recalled for further evaluation of serum thyroid-stimulating hormone (TSH) and free thyroxine 4 (FT4) by Chemiluminescence microparticle immunoassay (Abbot Park, IL 60064, USA). Diagnosis of CH was based on increasing levels of serum TSH (reference range: 0.30~4.80μIU/mL) and decreasing levels of serum FT4(reference range: 9.01~19.05pmol/L). In addition, a thyroidscintiscan and/or ultrasound were performed to confirm whether the developmental condition of thyroid was normal or not. The study was approved by the Ethics Committee of Shengjing Hospital of China Medical University.

Next-generation sequencing

By using TIANamp Blood DNA Mini Kit (TianGen, Beijing, China) Genomic DNA (gDNA) was extracted from peripheral blood leukocytes according to the manufacture's protocol. A total of 10ng DNA from each patient was sheared by using a sonicator (Covaris S220, NY, USA). The 150~250bp length fragments were selected. Paired-end DNA sequencing library was constructed according to the manufacture's protocol (Agilent, CA, USA). Briefly, adapter-modified gDNA fragments were enriched by six cycles of PCR amplification. The SureSelect Human All Exon V5 Kit (Agilent, CA, USA) was used to carry out the whole exome capture. Finally, 50Mb of gDNA fragments of the whole coding regions and flanking intronic

regions for DUOX2 gene were captured. After evaluating of quality control,samples were sequenced on Illumina Hiseq X ten platform according to the manufacture's instructions.

Bioinformatics analysis

The original image data obtained from NGS platform was converted into sequencing reads via base calling of Illumina CASAVA v1.8.2 software and then removed unusable reads by the quality control procedure. Sequencing reads were aligned to the human reference genome (UCSC hg19) by using BWA with default parameters.The dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/), the 1000 Genomes Project (http://ftp.ncbi.nlm.nih.gov/) and the Exome Sequencing Project (http://evs.gs.washington.edu/EVS/) were used to screen out the gene mutations with minor allele frequency (MAF) <0.05. SIFT, PolyPhen-2 and Mutation Taster were used to predict the pathogenicity of nonsense mutations and missense mutations and SWISS-MODEL was used for homology modeling of deletion mutations and frame-shift mutations. HMGD was searched to evaluate if the mutations were novel or known. Finally, American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG/AMP)8 were used to classified the pathogenic levels of mutations interested by the. In addition, a cohort of 200 ethnicity- matched healthy subjects was used to assess the variant frequencies in normal control. All control subjects had the normal TSH and FT4 levels.

Results

Statistical data of targeted sequencing

Among all targeted amplicons, average sequencing depth on target was 108.75x and the average coverage of target region was 98.80%, besides, 97.62% achieving actual reading depth over 4x, 94.97% achieving actual reading depth over 10x, and 89.72% achieving actual reading depth over 20x. This data indicated that NGS was in high accuracy.

Analysis of next-generation sequencing results

NGS analysis of DUOX2 revealed 20 different variants in 26 patients. The detected variants included ten missense mutations (p.A1131S, p.P76L, p.L1343F, p.R1211H, p.V779M, p.R1110Q, p.R885L, p.R683L, p.E805K, p.R422H), four nonsense mutations (p.W734X, p.K530X, p.Q573X, p.R625X), three deletion mutations (p.1160del, p.434_440del, p.336_337del) and three frame-shift mutations (p.T1107fs, p.Q202fs, p.K628fs), and the prevalence of DUOX2 mutation was up to 30.2% (26/86). Out of the 26 CH patients, 14 were found with single variation (including 13 heterozygous variation and one homozygous variation), 10 with two heterozygous variants and 2 patients were with three heterozygous variants. The variants p.K530X, p.L1343F and p.R1110Q were highly recurrent in our patient cohort: p.K530X occurring in six heterozygotes, p.L1343F occurring in five heterozygotes, and p.R1110Q occurring in three heterozygotes and one homozygotes.

These 20 variants were not detectable in 200 control individuals and were also absent in public population databases such as the dbSNP, 1000 Genomes Project databases or Exome Sequencing Project (ESP). The pathogenicity of these 20 variants was analyzed by SIFT, PolyPhen-2 and Mutation Taste (Table 1) and classified the pathogenic levels of variants according to assessment by using the ACMG/AMP guideline (Table 2 and Table 3).

Two rare variants were detected in our study, c.1007_1009del (p.336_337del) and c.3321delC (p.T1107fs), which were predicted as likely pathogenic variants by the ACMG/AMP guideline. We built homology modeling of them by SWISS- MODEL and found that the variant p.336_337del resulted in an obvious change of protein structure, and the variant p.T1107fs turned out to be a short protein structure (Figure 1).They all suggested that the amino acid deletions might be pathologic variants. We were unable to perform mutation segregation with phenotype within the family due to the lack data of immediate family members.

Genotype-phenotype correlation analysis

The clinical feature and laboratory test results of 26 patients with DUOX2 gene mutations were summarized in Table 4. All patients were unrelated individuals and diagnosed with CH by NBS.Out of 86 patients, 15 showed thyroid agenesis; 4 showed thyroid hypoplasia; 4 had increased size of thyroid gland; 2 had decreased size of thyroid gland; 1 showed thyroid ectopy. The levothyroxine sodium (L-T4) replacement therapy was started immediately after clinical diagnosis and the dose was adjusted according to the serum TSH and F4 levels. We found no correlation between genotypes and clinical phenotypes.The relationship between TSH level,FT4 level, initial dose of L-T4 and the accumulation of defects in DUOX2 also showed no significant association(Figure 2).

Discussion

Congenital hypothyroidism (CH) is the most common endocrine disorder that results in an abnormal nervous system development. A series of harmful consequences will happen if not diagnosed and treated early. Nowadays the routine newborn screening and the replacement therapy of L-T4 have greatly reduced the incidence of growth retardation and intellectual disability associated with CH. Although the pathogenesis of CH is unclear, thyroid dysgenesis (85%~90%) and thyroid dyshormomogenesis (10%~15%) are thought to be the most important causes leading to CH. It's reported that most thyroid dysgenesis cases are sporadic, and only about 5% cases have genetic origin[3], however, most thyroid dyshormomogenesis cases are caused by genetic factors and a decrease in H_2O_2 generation due to DUOX2 gene mutations has been reported to be one of the important causes of thyroid dyshormonogenesis[5].

Since the first DUOX2 mutations were described in patients with CH in 2002[9], dozens of DUOX2 mutations have been reported. However,the mutational spectrum and the genotype-phenotype correlations have not been fully established. Most patients with alternations in DUOX2 gene were either

caused by monoallelic or biallelic mutations, leading to a wide range of phenotypes, from normal thyroid to goiter[10], from a mild SCH to severe PCH[11]. In the present study, we conducted DUOX2 gene panel screening in a cohort of 86 patients with CH who were identified by newborn screening. The results showed that 30.2% (26/86) patients in North-East of China had at least one potential pathogenic variant. This rate is similar to the published studies among the Chinese (29%), in Japanese (43%) and Italian (45.5%), but is higher than the rate of 14.9% and lower than that of 83% reported for Chinese Han patients with CH[11,13-16]. Therefore, the prevalence of DUOX2 gene mutations varies in different targeted population. Maybe the methods involved in studies or some genetic diversity in different ethnic groups led to the differences. Our study identified twenty DUOX2 gene variants and sixteen of them were predicted to be pathogenic or likely pathogenic, and the other four were classified as uncertain significant variants by ACMG/AMP. The variants p.K530X (7%), p.L1343F (6%) and p.R1110Q (4.7%) were highly recurrent in our cohort. Among these three variants c.1588A>T (p.K530X) had a highest rate in our study and the result is in accordance with the previous studies in Asians including Chinese, Japanese, Korean and Malaysian[12,14,17,18]. Of all pathogenic variants predicted by ACMG/AMP, the variants p.R1110Q, p.R885L and p.1160del have been identified to be the important causes of CH. Jin and Narumi[12,13] et al. demonstrated in vitro experiments that the DUOX2 gene variants p.R1110Q, p.R885L and p.1160del significantly inhibited the H2O2-generation activity, and the above results are statistically significant. Thereby the three variants affected the synthesis of thyroxine and caused CH.

In addition, our study identified two rare and potentially pathogenic variants of DUOX2 gene namely, p.336_337del and p.T1107fs. From their homology modeling (Figure 1), we saw the protein structure either changed or obviously shortened, thereby leading to functional deficit in DUOX2. We also analyzed and compared the serum TSH levels, FT4 levels and initial dose of L-T4 between CH patients with three, two and one potential pathogenic variants of DUOX2 and found that the accumulation of defects in DUOX2 had no significant connection with the conditions regarding the serum TSH levels, FT4 levels and initial dose of L-T4 (Figure 2). Therefore, the relationship between DUOX2 genotypes and phenotypes is variable in different patients with CH. For example,the patient 49 with heterozygous DUOX2 variants (p.R683L and p.L1343F) appeared as thyroid agenesis but the patient 29 with the same variants showed a normal thyroid gland. We therefore conclude that the genotype-phenotype correlation is highly complex in CH patients.Maybe the environmental factors, ethnic differences, individual differences and some other candidate gene mutations associated in CH are responsible for the variable clinical phenotypes.

The limitations of this study include: first, our sample size was relatively small because we were very selective while recruiting study participants. Second, we didn’t undertake a comprehensive analysis of known genes other than DUOX2 associated with CH. Third, we didn’t carry out functional studies of the two rare variants. Finally, because of a lack of clinical data, we were unable to perform mutation segregation with phenotype within the family.

Conclusions

In summary, we conducted DUOX2 mutation screening and found a high prevalence of DUOX2 gene mutations in northeastern region of China. 30.2% (26/86) of CH patients were found with at least one potential pathogenic DUOX2 variants while 7% carried DUOX2 mutation p.K530X. We analyzed the relationship between the type and characteristics of DUOX2 variants and the clinical phenotype, as it is of great value to further understand the pathogenesis of CH. However, the genotype- phenotype correlation was extremely complex. In the future, functional studies should be carried out of on the DUOX2 pathogenic variants.

Abbreviations

ACMG, the American College of Medical Genetics and Genomics;

AMP, the Association for Molecular Pathology;

CH, congenital hypothyroidism;

DUOX2, dual oxidase 2;

DUOXA2, dual oxidase maturation factor 2;

ESP, Exome Sequencing Project;

FT3, free triiodothyronine;

FT4, free thyroxine 4;

LOF, loss of function;

L-T4, the levothyroxine sodium;

MAF, minor allele frequency;

NGS, next-generation sequencing;

NKX2-5, NK2 transcription factor related locus 5;

PAX8, the paired box gene 8;

PCH, permanent congenital hypothyroidism;

SCH, subclinical congenital hypothyroidism;

TG, thyroglobulin;

TPO, thyroperoxidase;

TSH, thyroid-stimulating hormone;

TSHR, thyroid-stimulating hormone receptor;

TTF-1, the thyroid transcription factors 1;

TTF-2, the thyroid transcription factors 2;

Declarations

Ethics approval and consent to participate

Written informed consents for publication of clinical details were obtained from the parents of any participant under the age of 16. The study was approved by the Ethics Committee of Shengjing Hospital of China Medical University.

Consent for publication

Written informed consents for publication of clinical details were obtained from the parents of any participant under the age of 18.

Availability of data and materials

Not applicable

Competing interests

The authors declare that they have no competing interests

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Authors' Contributions

All authors participated in concept and design, acquisition of data, analysis and interpretation of the data and review of the manuscript. CXY and LJH conducted the experiments; LY and QXS supplied critical reagents, serum samples and equipments; CXY, LJH and QXS wrote the manuscript, all authors read and approved the manuscript of the version to be published.

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Tables

Table 1 Prediction result of 20 variants

Variant		SIFT	PolyPhen-2	Mutation Taster	Result
c.3391G>T	p.A1131S	Deleterious	Probably damaging	Disease causing	Damage
c.2202G>A	p.W734X	Deleterious	Not available	Disease causing	Damage
c.227C>T	p.P76L	Tolerated	Possibly damaging	Disease causing	Tolerated
c.1588A>T	p.K530X	Tolerated	Not available	Disease causing	Tolerated
c.4027C>T	p.L1343F	Deleterious	Possibly damaging	Disease causing	Damage
c.3632G>A	p.R1211H	Deleterious	Probably damaging	Disease causing	Damage
c.2335G>A	p.V779M	Tolerated	Benign	Disease causing	Tolerated
c.3478_3480del	p.1160del	Not available	Not available	Not available	Not available
c.3329G>A	p.R1110Q	Deleterious	Possibly damaging	Disease causing	Damage
c.2654G>T	p.R885L	Not available	Probably damaging	Disease causing	Damage
c.2048G>T	p.R683L	Deleterious	Probably damaging	Disease causing	Damage
c.1717C>T	p.Q573X	Deleterious	Not available	Disease causing	Damage
c.2413G>A	p.E805K	Tolerated	Possibly damaging	Disease causing	Tolerated
c.1300_1320del	p.434_440del	Not available	Not available	Not available	Not available
c.3321delC	p.T1107fs	Not available	Not available	Not available	Not available
c.1007_1009del	p.336_337del	Not available	Not available	Not available	Not available
c.605_621del	p.Q202fs	Not available	Not available	Not available	Not available
c.1265G>A	p.R422H	Deleterious	Probably damaging	Disease causing	Damage
c.1883delA	p.K628fs	Not available	Not available	Not available	Not available
c.1873C>T	p.R625X	Tolerated	Not available	Disease causing	Tolerated

SIFT Sorting Intolerant from Tolerant, *PolyPhen-2* Polymorphism Phenotyping v2

Table 2 Classification and evidence of nonsense mutations and frameshift mutations

Variant		Level	PVS1	PS4	PM1	PM2		PP3
						1000 Genomes	ESP 6500	
c.2202G>A	p.W734X	Likely pathogenic	Yes	0	Not available	/	/	Damage
c.1588A>T	p.K530X	Pathogenic	Yes	0	Hame pero	0.000599042	/	Tolerated
c.1717C>T	p.Q573X	Pathogenic	Yes	0	Hame pero	/	/	Damage
c.1873C>T	p.R625X	Likely pathogenic	Yes	0	Not available	/	/	Tolerated
c.3321delC	p.T1107fs	Likely pathogenic	Yes	0	Not available	/	/	Not available
c.605_621del	p.Q202fs	Likely pathogenic	Yes	0	Not available	/	/	Not available
c.605_621del	p.K628fs	Likely pathogenic	Yes	0	Not available	/	/	Not available

PVS pathogenic very strong, *PS* pathogenic strong, *PM* pathogenic moderate, *ESP* Exome Sequencing Project, *PP* pathogenic supporting

Table 3 Classification and evidence of missense mutations and deletion mutations

Variant		Level	PS3	PS4	PM1	PM2		PM4	PP3
						1000 Genomes	ESP 6500		
c.3391G>T	p.A1131S	Uncertain significance	/	0	Not available	/	/	No	Damage
c.227C>T	p.P76L	Uncertain significance	/	0	Hame pero	/	/	No	Tolerated
c.4027C>T	p.L1343F	Likely pathogenic	/	0	FAD binding	0.000998403	0.0002	No	Damage
c.3632G>A	p.R1211H	Likely pathogenic	/	0	Not available	/	0.0002	No	Damage
c.2335G>A	p.V779M	Uncertain significance	/	0	Not available	0.000998403	/	No	Tolerated
c.3329G>A	p.R1110Q	Pathogenic	Damage	0	Not available	0.000399361	/	No	Damage
c.2654G>T	p.R885L	Pathogenic	Damage	0	EF-2	0.000798722	/	No	Damage
c.2048G>T	p.R683L	Likely pathogenic	/	0	Not available	0.000599042	/	No	Damage
c.2413G>A	p.E805K	Uncertain significance	/	0	Not available	/	/	No	Tolerated
c.1265G>A	p.R422H	Likely pathogenic	/	0	Hame pero	0.000599042	/	No	Damage
c.3478_3480del	p.1160del	Likely pathogenic	Damage	0	Helical	/	/	Yes	Not available
c.1300_1320del	p.434_440del	Likely pathogenic	/	0	Hame pero	/	/	Yes	Not available
c.1007_1009del	p.336_337del	Likely pathogenic	/	0	Hame pero	/	/	Yes	Not available

PS pathogenic strong, *PM* pathogenic moderate, *ESP* Exome Sequencing Project, *PP* pathogenic supporting

Table 4 Clinical features, laboratory results of 26 CH patients with DUOX2 gene mutations

Patient NO.	Age[a] (year)	Sex	Results of thyroid ultrasonography	FT4[a] (pmol/L)	TSH[a] (μIU/mL)	DUOX2 variant
1	Newborn screening	Female	Normal	<5.15	>100	c.3391G>T(p.A1131S) c.2202G>A(p.W734X) c.227C>T(p.P76L)
2	Newborn screening	Female	Normal	8.31	31.82	c.227C>T(p.P76L) c.1588A>T(p.K530X)
3	Newborn screening	Male	Normal	<5.15	>100	c.4027C>T(p.L1343F)
4	Newborn screening	Female	Normal	6.71	27.08	c.3632G>A(p.R1211H)
5	Newborn screening	Male	Agenesis	<5.15	>100	c.2335G>A(p.V779M) c.3478_3480del (p.1160_1160del)
6	Newborn screening	Female	Normal	7.34	10.255	c.3329G>A(p.R1110Q)
7	Newborn screening	Female	Normal	8.24	17.908	c.4027C>T(p.L1343F) c.1588A>T(p.K530X)
8	Newborn screening	Male	Normal	<5.15	>100	c.2654G>T(p.R885L)
9	Newborn screening	Female	Normal	7.47	70	c.2048G>T(p.R683L) c.4027C>T(p.L1343F)
10	Newborn screening	Female	Normal	5.13	>100	c.4027C>T(p.L1343F)
11	Newborn screening	Female	Decreased	<5.15	>100	c.1717C>T(p.Q573X)
12	Newborn screening	Male	Increased	8.68	23.119	c.3329G>A(p.R1110Q)
13	Newborn screening	Male	Normal	<5.15	>100	c.2413G>A(p.E805K)
14	Newborn screening	Female	Normal	6.35	70	c.1588A>T(p.K530X) c.1300_1320del (p.434_440del)
15	Newborn screening	Male	Normal	<5.15	>100	c.605_621del(p.Q202fs)
16	Newborn screening	Male	Normal	<5.15	>100	c.3632G>A(p.R1211H)
17	Newborn screening	Female	Agenesis	5.95	46.906	c.2048G>T(p.R683L) c.4027C>T(p.L1343F) c.3632G>A(p.R1211H)
18	Newborn screening	Female	Normal	6.63	>100	c.1588A>T(p.K530X)
19	Newborn screening	Male	Normal	6.48	>100	c.3478_3480del (p.1160_1160del) c.1588A>T(p.K530X)
20	Newborn screening	Female	Normal	7.43	>100	c.1588A>T(p.K530X)
21	Newborn screening	Female	Normal	<5.15	>100	c.1265G>A(p.R422H)
22	Newborn screening	Male	Increased	7.17	>100	c.1883delA(p.K628fs) c.3321delC(p.T1107fs)
23	Newborn screening	Male	Normal	8.46	59.05	c.227C>T(p.P76L) c.1007_1009del (p.336_337del)
24	Newborn screening	Male	Normal	<5.15	>100	c.3329G>A(p.R1110Q)
25	Newborn screening	Male	Normal	<5.15	>100	c.605_621del(p.Q202fs) c.1873C>T(p.R625X)
26	Newborn screening	Female	Normal	0.3	86.14	c.3478_3480del (p.1160_1160del) c.3329G>A(p.R1110Q)

FT4 free thyroxine 4, *TSH* thyroid stimulating hormone, [a] Age, TSH, FT4 at diagnosis

Figures

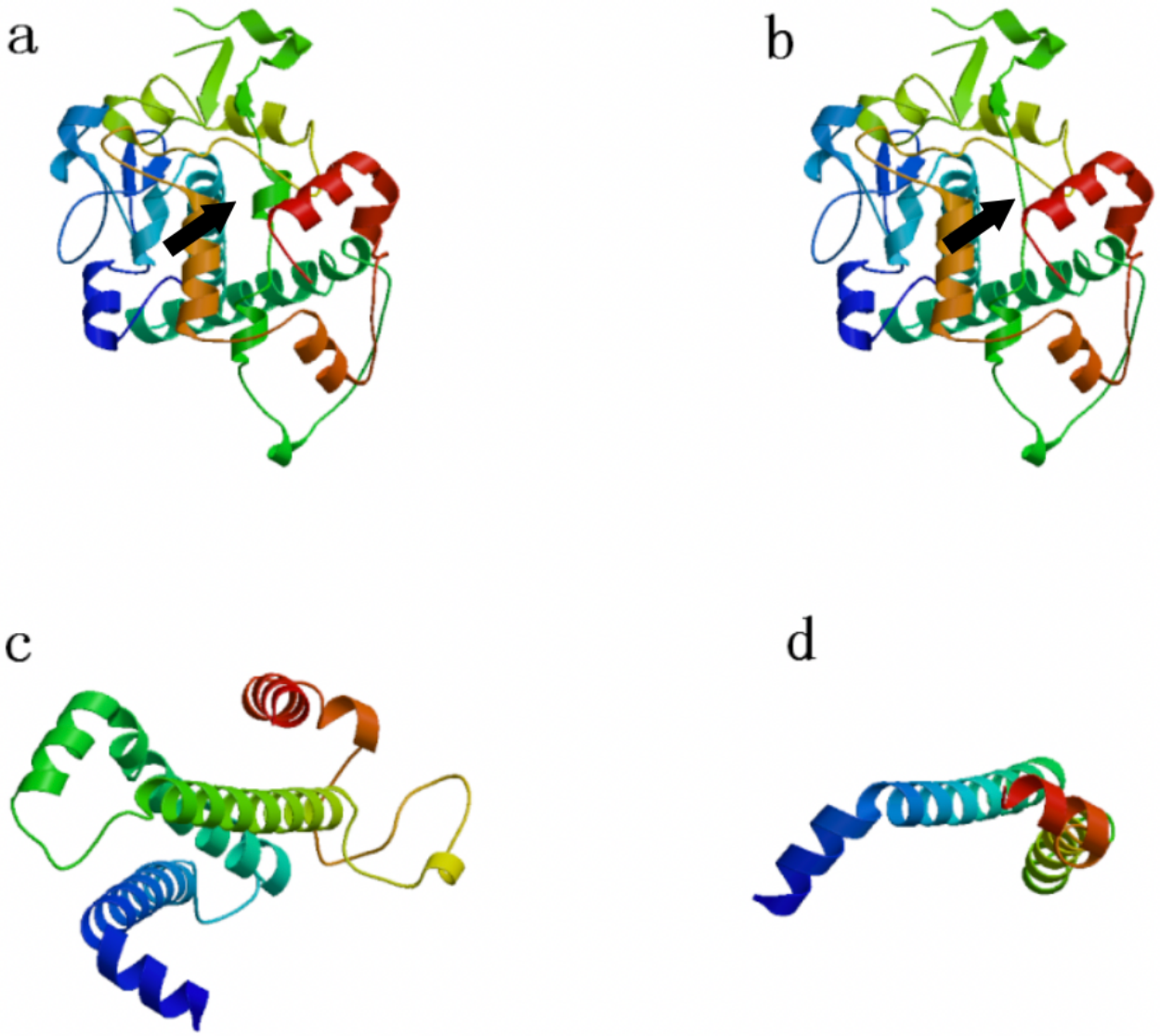

Figure 1

Protein structure models of two rare variations. a indicates the wild type of DUOX2; b indicates the mutant type of p.336_337del and the arrow indicates the deficient protein structure; c indicates the wild type of p.T1107fs; d indicates the mutant type of p.T1107fs.

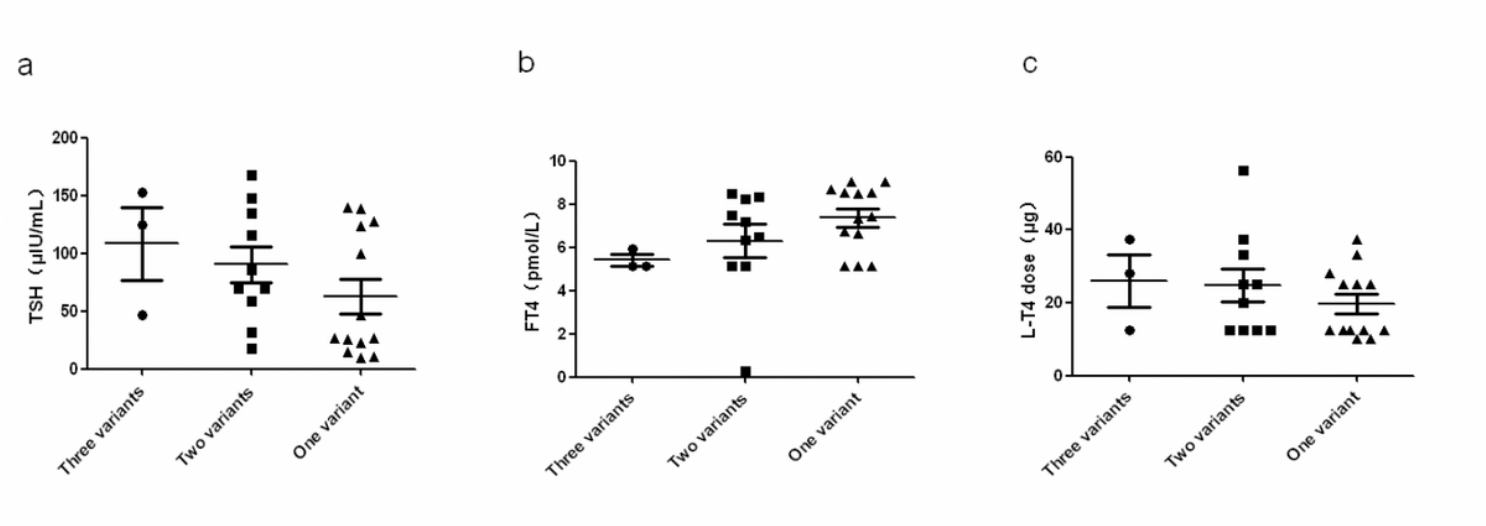

Figure 2

(a,b and c); Analysis and comparison of TSH levels, FT4 levels and initial dose of L-T4 between patients with one, two and three DUOX2 variants.