Clinica Chimica Acta 458 (2016) 30–34

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Next-generation sequencing analysis of *DUOX2* in 192 Chinese subclinical congenital hypothyroidism (SCH) and CH patients

Chunyun Fu [a,b,1], Shiyu Luo [a,b,1], Shujie Zhang [a,b], Jin Wang [a,b], Haiyang Zheng [a,b], Qi Yang [a,b], Bobo Xie [a,b], Xuyun Hu [a,b], Xin Fan [a,b], Jingsi Luo [a,b], Rongyu Chen [a,b], Jiasun Su [a,b], Yiping Shen [a,c], Xuefan Gu [d,*], Shaoke Chen [a,b,**]

[a] Department of Genetic Metabolism, Children's Hospital, Maternal and Child Health Hospital of Guangxi Zhuang Autonomous Region, Nanning 530003, People's Republic of China
[b] GuangXi Center for Birth Defects Research and Prevention, Nanning 530003, People's Republic of China
[c] Boston Children's Hospital, Harvard Medical School, Boston 02115, MA, United States
[d] Endocrinology and Genetic Metabolism of Institute for Pediatric Research, Xinhua Hospital Affiliated to Shanghai Jiaotong University School of Medicine, Shanghai 200092, People's Republic of China

ARTICLE INFO

Article history:
Received 25 February 2016
Received in revised form 17 April 2016
Accepted 17 April 2016
Available online 21 April 2016

Keywords:
Subclinical congenital hypothyroidism
Dual oxidase 2 gene (DUOX2)
Gene mutations
Next-generation sequencing
Chinese population

ABSTRACT

Background: Defects in the human dual oxidase 2 (*DUOX2*) gene are reported to be one of the major causes of congenital hypothyroidism (CH). This study was set to examine the *DUOX2* mutation spectrum and prevalence among Chinese CH and subclinical congenital hypothyroidism (SCH) patients and to define the relationships between *DUOX2* genotypes and clinical phenotypes.
Methods: Peripheral venous blood samples were collected from 192 CH/SCH patients in Guangxi Zhuang Autonomous Region of China. All exons and their exon-intron boundary sequences of the 11 known CH associated genes including *DUOX2* were screened by next-generation sequencing (NGS).
Results: NGS analysis of *DUOX2* revealed 18 rare non-polymorphic variants in 57 CH/SCH patients. Sequencing of other CH candidate genes in the 57 patients revealed 2 thyroglobulin (*TG*) variants. All variants included 11 known mutations, 8 novel variants in *DUOX2* and one novel variant in *TG*, among which three variants p.K530X, p.L1343F and p.R683L are highly recurrent in our patient cohort. 35 (83%) of the 42 patients with one or two *DUOX2* pathogenic variants turned out to be SCH or transient congenital hypothyroidism (TCH), whereas 13 (87%) of the 15 patients with three or more *DUOX2* pathogenic variants are associated with permanent congenital hypothyroidism (PCH). The accumulation of defects in *DUOX2* contribute to the more severe disease regarding thyroid stimulating hormone (TSH) levels, free thyroxine (FT4) levels and initial dose of L-thyroxine (L-T4).
Conclusion: Our study expanded the mutational spectrum of the *DUOX2* and *TG* genes and provided the best estimation of the *DUOX2* mutation rate (29%) for CH/SCH patients in Guangxi Zhuang Autonomous Region of China. Most one or two *DUOX2* pathogenic variants turned out to be SCH or TCH, whereas patients with three or more *DUOX2* pathogenic variants were mostly associated with PCH. The coexistence of multiple pathogenic variants may have contributed to the severity of the hypothyroid condition.

1. Introduction

Congenital hypothyroidism (CH) is the most common neonatal endocrine disorder in infancy with prevalence ranging from 1:2000 to 1:4000 [1,2]. It's reported that the most prevalent cause of CH worldwide is still iodine deficiency [3], however, considerable progress has been made to identify the genetic causes in CH patients. Based on genetic alterations, CH can be classified into two groups: 80%–85% of cases are caused by disorders of thyroid gland development (thyroid dysgenesis, comprising agenesis, ectopy, or hypoplasia) [4], which has been linked to gene mutations in thyroid-stimulating hormone receptor (*TSHR*), paired box gene 8 (*PAX8*), thyroid transcription factor 1 (*TTF1/NKX2.1*), thyroid transcription factor 2 (*TTF2/FOXE1*) and NK2 transcription factor related locus 5 (*NKX2.5*) [5]; the remaining 15%–20% of CH cases are caused by abnormalities in thyroid hormone synthesis (thyroid dyshormonogenesis), which is associated with the presence of goiter or with a eutopic gland of normal size [6]. Molecular studies have found that dyshormogenesis in CH is caused by defects in genes such as dual oxidase 2 (*DUOX2*), thyroglobulin (*TG*), thyroid peroxidase

* Corresponding author.
** Correspondence to: S. Chen, Department of Genetic Metabolism, Children's Hospital, Maternal and Child Health Hospital of Guangxi Zhuang Autonomous Region, Nanning 530003, People's Republic of China.
E-mail addresses: gu_xuefan@163.com (X. Gu), chenshaoke123@163.com (S. Chen).
[1] Chunyun Fu and Shiyu Luo contribute equally to this work.

http://dx.doi.org/10.1016/j.cca.2016.04.019
0009-8981/

(*TPO*), Pendrine (*SLC26A4*), dehalogenase 1 (*DEHAL1*) and sodium iodide symporter (*NIS*) [7].

Defects in the human *DUOX2* gene are reported to be one of the major causes of CH. The *DUOX2* gene is located on chromosome 15 and consists of 34 exons [8]. The *DUOX2* protein is a 1548-amino-acid polypeptide located at the apical membrane of thyrocytes and generates the hydrogen peroxide (H_2O_2) needed by thyroid peroxidase for the incorporation of iodine into thyroglobulin, an essential step in thyroid hormone synthesis. Up to date, the mutational spectrum of the *DUOX2* gene and the phenotype-genotype correlations have not yet fully been established. Both biallelic and monoallelic *DUOX2* mutations lead to a wide spectrum of phenotypes, ranging from mild subclinical congenital hypothyroidism (SCH) with elevated thyroid stimulating hormone (TSH) levels but normal thyroid hormone levels, to overt CH with thyroid dyshormonogenesis. Also, little is known about *DUOX2* mutation spectrum and its prevalence among Chinese CH/SCH patients. In our recent study, we identified *DUOX2* pathogenic variants in 13 of 45 cases (29%) and found that most monoallelic or biallelic *DUOX2* pathogenic variants turned out to be TCH, while patients with triallelic *DUOX2* pathogenic variants were associated with permanent congenital hypothyroidism (PCH) [9]. The aim of this study was to screen for the presence of mutations in *DUOX2* gene among patients with CH/SCH in China, to define the relationships between *DUOX2* genotypes and clinical phenotypes and to confirm our previous findings based on a larger sample size and comprehensive analysis of all known CH associated genes.

2. Methods

2.1. Patients

A total of 192 newborns were enrolled in this study (the previous 45 CH patients collected for *DUOX2* mutation screening by Sanger sequencing were not included in this study [9]), including 140 patients with CH and 52 patients with SCH, who were identified through a large-scale newborn screening (NBS) covering 615,000 cases in Guangxi, China, from July 2009 to June 2013. CH NBS were done with filter paper between 72 h and 7 days after birth. Blood samples were collected from the heel and the TSH level were measured by time-resolved fluorescence assay (Perkin Elmer, USA). Subjects with increased TSH levels (TSH ≥ 8 mIU/l) during NBS were followed-up for further evaluation. Serum TSH and free thyroxine (FT4) were determined by electrochemiluminescence assay (Cobas e601, Roche Diagnostics, USA). Diagnosis of CH was based on elevated TSH levels (TSH ≥ 10 mIU/l) and decreased FT4 levels (FT4 < 12 pmol/l). Patients with elevated TSH levels and normal FT4 levels (normal range 12–22 pmol/l) were diagnosed as SCH. Permanent or transient CH was determined using results of thyroid function tests after temporary withdrawal of L-thyroxine (L-T4) therapy at approximately 2 years of age. Thyroid ultrasonography and ^{99m}Tc scintigraphy were performed during the neonatal period before treatment.

This study was approved by the local Medical Ethics Committee. Informed consent was obtained from the parents of the patients.

2.2. Next generation sequencing and bioinformatics analysis

Peripheral venous blood samples were collected from the patients. Genomic DNA was extracted from peripheral blood leukocytes using QIAamp DNA Blood Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. CH capture panel as Illumina Truseq Custom Amplicon v1.5 kit was designed and 11 known CH associated genes (*DUOX2*, *TSHR*, *PAX8*, *NKX2.1*, *NKX2.5*, *FOXE1*, *TG*, *TPO*, *NIS*, *SLC26A4* and *DEHAL1*) were included with the whole coding regions and flanking intronic sequences. The prepared sample library was sequenced by Illumina MiSeq instrument using MiSeq Reagent Kit v2, 500-cycles (Illumina Inc., San Diego, CA). Illumina Amplicon Viewer v1.3 and MiSeq Reporter v2.3 software were used for data analysis, and the SnpEff [10] was used for variant annotation. SIFT [11] and MutationTaster [12] were used to evaluate the pathogenicity of the novel variants. Multiple sequence alignment of the DUOX2 family protein among different species were carried out by DNAMAN software version 8 to analyze the amino acid conservation of the mutated sites. All variants were further validated by Sanger sequencing. In addition, a cohort of 400 ethnicity-matched healthy subjects with normal FT4 and TSH levels were sequenced to assess the variant frequencies in normal control population.

3. Results

Next generation sequencing analysis of *DUOX2* revealed 18 different variants in 57 individuals (47 CH and 10 SCH patients) including 31 with single heterozygous variation, 11 with two variants and 15 with three or more variants. Sequencing of other CH candidate genes in the 57 patients with *DUOX2* mutations further revealed 2 *TG* variants. All variants were confirmed by Sanger sequencing (Figs. S1, S2). The variants included 11 known mutations: c.3329G > A (p.R1110Q), c.1588A > T (p.K530X), c.2635G > A (p.E879K), c.3340delC (p.L1114SfsX56), c.903G > T (p.W301C), c.3413C > A (p.A1138D), c.1736T > C (p.L579P), c.2048G > T (p.R683L), c.2524C > T (p.R842X), c.4027G > T (p.L1343F) in *DUOX2* and c.8119C > T (p.R2707X) in *TG*, as well as 9 novel variants: two in-frame deletions c.2102–2104delGAG (p.G702del) and c.3478–3480delCTG (p.L1160del), six missense variants c.3251G > A (p.R1084Q), c.4000C > T (p.R1334W), c.3967G > A (p.A1323T), c.4475G > A (p.R1492H), c.3391G > T (p.A1131S), c.244C > A (p.R82S) in *DUOX2* and one nonsense mutation c.5766C > A (p.Y1922X) in *TG* (Fig. 1). The variants p.K530X, p.R683L, p.L1343F are highly recurrent in our patient cohort: p.K530X occurring in fourteen heterozygotes and one homozygote, p.R683L in six heterozygotes and ten homozygotes, and p.L1343F in nineteen heterozygotes.

The novel truncating variant p.Y1922X was not detected in 400 control individuals and also absent from public population databases such as 1000 Genomes Project. It was classified as pathogenic variants (PVS1 + PS4) according to our assessment using the ACMG/AMP guideline [13].

The other novel variants were not detected in our 400 control individuals. Both the identified novel missense variants and in-frame deletions were found to be located in the highly conserved regions of DUOX2 (Fig. 2). SIFT and MutationTaster predicted that the novel missense variants likely had deleterious effects by damaging DUOX2 function. The two novel deletions (c.2102–2104delGAG and c.3478–3480delCTG) both removed 3 nucleotides, resulting in an in-frame deletion of Glycine 702 in the Topological domain and an in-frame deletion of Leucine 1160 in the Transmembrane area, respectively. Those all suggested that the amino acid substitutions/deletions might be pathologic. Because of a lack of data, we were unable to perform mutation segregation with phenotype within the family.

The clinical features and laboratory test results were summarized in Table S1. All patients were born at full-term to unrelated parents and diagnosed with CH/SCH by NBS. L-T4 replacement therapy was started immediately after clinical diagnosis and the dose was adjusted according to the serum TSH and FT4 levels. After temporary withdrawal of L-T4 therapy at approximately 2 years of age, 35 (83%) of the 42 patients with one or two *DUOX2* pathogenic variants turned out to be SCH or transient congenital hypothyroidism (TCH), four of them turned out to be permanent congenital hypothyroidism (PCH), and the other three patients were currently under two years of age thus still remained to be determined. By contrast, 13 (87%) of the 15 patients with three or more *DUOX2* pathogenic variants are associated with PCH and the other two of them turned out to be TCH. Most patients with one or two *DUOX2* pathogenic variants turned out to be SCH or TCH, whereas patients with three or more *DUOX2* pathogenic variants were mostly associated with