Novel non-synonymous mutations of PAX8 in a cohort of Chinese with congenital hypothyroidism

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Abstract

Background: The transcription factor paired box 8 (PAX8) was associated with type 2 congenital non-goitrous hypothyroidism (CHNG2), a clinical phenotype of congenital hypothyroidism (CH). Though studied in a few regions with different ethnicities, the incidence of PAX8 mutations varied, even among Chinese cohorts in different regions. This study aimed to identify and characterize PAX8 mutations and explore the prevalence of its mutations in another cohort of CH.

Methods: The 105 unrelated Chinese patients with CH were collected from four major hospitals. Exomes of the 105 samples were sequenced by Hiseq 2000 platform to identify mutations of PAX8 on genomic DNAs extracted from peripheral blood samples. Luciferase reporter assays were used to assess the effects of mutations on the transcription of thyroid peroxidase (TPO).

Results: Three PAX8 mutations in four subjects were identified in 105 samples. One variant, rs189229644, was detected in two subjects, and categorized as uncertain significance. The other two missense mutations (275T>C/Ile92Thr and 398G>A/Arg133Gln) were not detected in three large-scale genotyping projects, namely 1000 Genome Project, Exome Aggregation Consortium and GO Exome Sequencing Project. Functional studies for the two mutations revealed that they could impair the transcription ability of PAX8 on one of its target genes, TPO. Therefore, the two mutations were causative for the pathogenesis of CHNG2. After combining the studies of PAX8 mutations, an average frequency of 1.74% (21/1209) could be obtained in Chinese patients with CH.

Conclusion: The study specifically demonstrates the role of two mutations in impairing the transcription ability of PAX8, which should be considered as pathogenic variants for CH.

Keywords: Congenital hypothyroidism; Paired box 8; Novel non-synonymous mutation; Transcription factor

Introduction

Congenital hypothyroidism (CH) is the most common condition of thyroid hormone deficiency present at birth and preventable against mental and growth retardation. It had been reported that the incidence of CH has progressively increased, with a reported incidence at 1:1400 to 1:2800.1 Mainly, there exist two major types of the endocrine disease: (1) congenital non-goitrous hypothyroidism (CHNG), caused by the defects in the development and migration of the thyroid gland; and (2) thyroid dyshormonogenesis (TDH), caused by genetic defects of proteins involved in thyroid hormone synthesis [Supplementary Table 1, http://links.lww.com/CM9/A29]. It has been generally accepted that up to 75% to 85% of CH cases are due to CHNG, which leads to complete absence of the thyroid gland (athyreosis, 35%–40%), a normally located but small thyroid (hypoplasia, 5%), and an abnormally located thyroid gland (ectopy, 30%–45%).1,2 About 15% to 20% are due to TDH,2,3 in which patients harbor inborn genetic defects in one of the steps for thyroid hormone synthesis in thyrocytes.4 Most of the untreated patients with CH with TDH are associated with goitrous enlargement of the thyroid gland. Till now, seven genes have been linked with the occurrence of TDH, such as SLC5A5/NIS, SLC26A4/PDS, thyroid peroxidase (TPO), dual oxidase 2 (DUOX2),

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Besides, there also exist rare syndromic diseases with CH, such as Bamforth-Lazarus syndrome, neonatal diabetes mellitus, and congenital hypothyroidism (NDH) syndrome, central hypothyroidism and testicular enlargement (CHTE) and choreoathetosis, CH with or without pulmonary dysfunction (CAHTP; also referred to as brain-lung-thyroid syndrome). It has been reported that some thyroid transcription factors are essential for thyroid organogenesis, paired box 8 (PAX8) is one of these important factors.

PAX8 gene contains 12 exons and spans a genomic region of 63 kb in length. It encodes a member of the PAX family of transcription factors that contain a PAX domain and a paired-box protein 2 C terminal (Pax2-C). The PAX domain is responsible for DNA binding. As highly expressed in thyroid gland, PAX8 is involved in the development of thyroid follicular cell and expression of thyroid-specific genes. Mutations in PAX8 have been associated with thyroid follicular carcinomas, atypical follicular thyroid adenomas, and thyroid dysgenesis (ie, athyrrosis, hypoplastic, or ectopic thyroid gland). After curating reported mutations of PAX8, the causal variants are prevalently located in the PAX domain, thus impairing the DNA binding ability.

The prevalence of PAX8 pathogenic mutations differs significantly among patients with CH in different ethnic populations. It has been reported that mutations of PAX8 were detected in 8.4% of French patients with CH, whereas only 0.5% of patients with CH was reported among Czechs. According to the genetic screening reports among CH cohorts in China, the prevalence of PAX8 pathogenic variants varied in different provinces. For example, a prevalence of 3.38% was reported in Guangxi, 2.73% in Shanghai, and 1.14% in Shandong province. The average incidence of PAX8 mutations was 1.74% in China [Supplementary Table 2, http://links.lww.com/CM9/A29].

Therefore, it has been noticed that the genetic molecular pathogenesis of PAX8 has not been uniformly documented and characterized in different populations, especially for Chinese population. To have a comprehensive knowledge of the PAX8 mutations among Chinese patients with CH, this study performed genetic screening for PAX8 in a large CH cohort from Northeastern of China and functionally characterized the two identified novel mutations.

**Methods**

**Ethical approval**

The study was conducted in accordance with the *Declaration of Helsinki* and was approved by the Medical Ethics Committee of Reproductive Health Hospital of Xinjiang Uygur Autonomous Region. Informed written consent was obtained from the patients’ parents prior to their enrollment in this study.

**Subjects**

The 105 sporadic patients with CH (including 40 females and 65 males; mean age: 1.8 ± 0.5 years) were enrolled in this study. Most of patients were initially identified by newborn screening from September 2010 to July 2016 and followed up in Pediatric Clinics in the four hospitals (Xijing Hospital, Tangdu Hospital, Guanghang People’s Hospital, and Reproductive Health Hospital of Xinjiang Uygur Autonomous Region). Newborn screening was performed with dried blood filter paper for CH between 72 h and 7 days after birth. Blood samples were collected from the heel and thyroid-stimulating hormone (TSH) level measured by time-resolved fluorescence assay (Auto DELFI A 1235; PerkinElmer, Wellesley, MA, USA). Subjects with increased TSH (≥8 mU/L) levels were recalled for further evaluation.

**Genomic DNA extraction**

Genomic DNAs were extracted from peripheral blood leukocytes using the QIAamp DNA blood kit (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. The quantity of DNA was measured by NanoDrop 2000 microvolume spectrophotometer (Thermo Fisher, Waltham, MA, USA).

**Exome sequencing for CH samples**

A total amount of 5 μg genomic DNA of each sample was fragmented by Covaris S220 (Thermo Fisher). Both ends of the resulting fragments were ligated with adapters. The ligated DNAs were then amplified by ligation-mediated PCR (LM-PCR), purified, and hybridized to the NimbleGen 44 M human exome array for enrichment. High-throughput sequencing for each captured library was performed on Hiseq 2000 platform (Illumina, San Diego, CA, USA). Each sample was sequenced at the mean depth of 100× to achieve high sensitivity and accuracy for mutations detection. Raw image files were processed by Illumina basecalling Software 1.7 for base-calling with default parameters.

**Variants identification and functional predictions**

The sequencing reads were mapped to the reference human genome (GRCh37, UCSC hg19) using BWA (http://bio-bwa.sourceforge.net/). Single-nucleotide variants (SNVs) and insertions-deletions (indels) were identified using the SAM tools (http://samtools.sourceforge.net/), based on filtered variants with a mapping quality score ≥20, and annotated using ANNOVAR (http://www.openbioinformatics.org/annovar/). Mutations identified by exome
Samples HEK293 cells were cultured in Dulbecco modified Eagle medium supplemented with 1% penicillin, 1% streptomycin and 10% fetal bovine serum at 37°C with 5% CO₂. When cells confluence reached 90%, transfection was performed using Lipofectamine 2000 (Thermo Fisher) for wild type or mutant PAX8 plasmids together with pGL3-PrPO and pRL-TK (Promega, Fitchburg, WI, USA), following the manufacturer’s instructions. After 48 h of transfection, Dual-Glo Luciferase assay system (Promega) was used to detect the signals produced by firefly luciferase and renilla luciferase. The ratio between measured firefly and Renilla luciferase activities was expressed relative to the ratio obtained in cells transfected with reporter and empty expression vector (pcDNA3.1). All transfections were performed in triplicate and three independent experiments were similarly performed.

Results

Identification of novel PAX8 mutations

Four variants of PAX8 with a minor allele frequency (MAF) <5% were in detected in four individuals among the 105 CH samples [Table 1]. According to the sequencing data, two of four samples only had variants in PAX8 gene. One patient carried mutations in four known causal genes for CH (PAX8, TG, TPO, and DUOX2) and one patient in two known genes (PAX8 and TG) [Table 1]. The minor allele frequencies of the five SNPs were extracted from East Asian populations (n = 1008) of 1000 Genome Project, Exome Aggregation Consortium (n = 60,706) and GO Exome Sequencing Project (GO-ESP) (n = 6503) [Table 2]. Two variants of TG were with a MAF >0.05 in the data of 1000 Genome Project (rs74590117) and NHLBI GO-ESP (rs10091530), and should be regarded as common polymorphisms. As for other three SNPs (rs114406277, rs144153950, and rs189229644), they were considered as rare variants with MAF <0.01. Predictions by Polyphen or SIFT for these three SNPs indicated benign or deleterious aspects without affirmative significance and should be categorized as “Uncertain.”

Except for the five known variants, two heterozygous missenses (c.275T>C/p.Ile92Thr and c.398G>A/p.Arg133Gln) were identified in PAX8 in two individual samples [Table 2]. One patient (Sample 3) with c.275T>C was a male subject, who was born at 41 weeks of gestation from non-consanguineous parents with birth weight of 3200 g and length of 52 cm. He was diagnosed as CH by neonatal screening with a high TSH (>100 μU/mL) and low FT4 (6.2 pmol/L). The other patient (Sample 4) with c.398G>A was a female subject, who was born at 38 weeks of gestation from non-consanguineous parents with birth weight of 12,000 g and length of 85 cm. The neonatal screening revealed a high TSH (97.1 μU/mL) and low FT4 (3.1 pmol/L).

These two mutations have not been discovered by any of the three large-scale genotyping projects. The two non-synonymous variants were not detected in 347 ethnic-matched healthy controls. Therefore, they were regarded as novel SNVs. Functional predictions by Polyphen and SIFT indicated that the two SNVs were detrimental to the proper functions of PAX8 [Table 2] and should be classified as pathogenic variants. These novel variants located at the PAX domain in which the majority of

| Samples | Genes with rare variants |
|---------|-------------------------|
| 1       | PAX8                    |
| 2       | TG                      |
| 3       | TPO                     |
| 4       | DUOX2                   |
| 1       | rs189229644             |
| 2       | rs74590117              |
| 3       | rs114406277             |
| 4       | rs144153950             |

Table 1: Samples with rare variants in the known causal genes of CH.
reported pathogenic variants located [Figure 1]. All variants were confirmed through MassArray iPLEX MALDI-TOF platform.

We also extracted the protein sequences of PAX8 of 12 different species from NCBI GenBank, including Homo sapiens (Human), Pan troglodytes (Chimpanzee), Pongo abelii (Sumatran orangutan), Macaca mulatta (Rhesus macaque), Mus musculus (Mouse), Rattus norvegicus (Rat), Bos taurus (Cattle), Ailuropoda melanoleuca (Giant panda), Puma concolor (Cougar), Ovis aries (Sheep), Cricetulus griseus (Chinese hamster), and Xenopus tropicalis (Western clawed frog). The sequences were aligned by CLUSTALW integrated in MEGA 4. It revealed that the two sites (Ile92Thr and Arg133Gln) were highly conserved in these species during evolution [Figure 2].

**Novel mutations affecting the transcription ability of PAX8**

Since PAX8 is a DNA-binding transcription factor, driving the expression of genes responsible for thyroid hormone synthesis, such as TPO and TG. The promoter region upstream of TPO (chr2: 1,416,733–1,417,232) was cloned into pGL3-basic, named as pGL3-P_TPO. The ORF of the longest isoform of PAX8 (NM_003466) was also introduced by DNA synthesis. After 48 h transfection with plasmids encoding wild type or mutant PAX8, together with pGL3-P_TPO and the internal control pRL-TK, the luciferase activities were measured. Comparing with wild type PAX8, the luciferase activities were significantly decreased in cells transfected with pGL3-P_TPO and mutant PAX8 (P < 0.029) [Figure 2D].

**Discussion**

In our cohort involving 105 patients with CH with Chinese origin, two novel missense mutations of PAX8 were identified and functionally characterized. The result of luciferase reporter assay revealed that the efficiency to drive TPO expression was severely impaired for PAX8 with Ile92Thr or Arg133Gln mutations.
It had been reported that the double knockout of PAX8 gene could result in thyroid aplasia in mice and heterozygous loss-of-function mutations of PAX8 led to various forms of thyroid dysgenesis in humans. According to literature reviews for studies about PAX8 mutations in CH samples, most of the function-detrimental mutations were located in the PAX domain [Figure 2], through which PAX8 binding with its DNA motifs. Although inherited through autosomal dominant transmission, the penetrance varied greatly for some PAX8 mutations. It is worth noting that the clinical phenotypes of individuals with heterozygous PAX8 mutations vary considerably within the same family.

The DNA binding sites of PAX8 had been proved at 34 amino acid positions of PAX domain (9–133 aa): 14, 15, 20, 22..24, 26, 31, 43, 45, 54, 57, 59, 60, 64, 73, 79, 81..84, 103..105, 124, 126, 127, 130, 133. Consistent with our current study, most of the mutations have been reported to be localized in the coding regions of PAX8, particularly in the PAX domain, which cause loss of function of PAX8 and lead to the appearance of CH in many ethnic populations. In our current cohort of Chinese patients with CH, we identified two novel PAX8 mutations Ile92Thr and Arg133Gln. Together with the other causative mutations of PAX8 in Shandong, Guangxi, and Shanghai, there were 21 samples with PAX8 mutations in a total of 1209 patients with CH, making an average prevalence at 1.74% [Supplementary Table 2, http://links.lww.com/CM9/A29]. This was consistent with the reported low prevalence of PAX8 mutations in thyroid dysgenesis in other ethnic populations. For example, in 17 different ethnic cohorts of patients with CH with European origins, the averaged frequency of PAX8 mutations was 1.0%, ranging from 0 to 3.4%. Even in Chinese populations, the prevalence of PAX8 pathogenic variants varied greatly in populations from different region, from 1.00% in Shandong to 2.73% in Shanghai.

Since Ile92Thr and Arg133Gln mutations identified in our cohort are located in the region of PAX8 responsible for the DNA binding, the amino acid substitutions might severely interfere with the expression of its target genes, such as TPO and TG. Luciferase reporter assay on TPO promoter revealed that for the two mutations could significantly reduce the expression of TPO gene.

In conclusion, novel mutations of PAX8 gene were identified in a cohort of unrelated Chinese patients with CH. The frequency of PAX8 mutations was 1.90% in our data, which was similar to other studies involving Chinese samples in different regions. The two mutations were functionally characterized by luciferase reporter assay and displayed obvious detrimental effects on the expression of TPO, one well-known target of PAX8. Thus the pathogenic roles of these two mutations were established in the development of CH from thyroid dysgenesis. This study documented the prevalence and functional characterization of PAX8 mutations in a large cohort of patients with CH from a new region of China.

Figure 2: Characterization of PAX8 and two mutations in our cohort of CH samples. (A) Diagram of PAX8 gene. Arrow indicates the transcription direction. (B) Diagram of PAX8 protein. (C) Multiple sequence alignment of PAX8 from 12 different species. (D) Luciferase reporter assay of the two mutations of PAX8. CH: Congenital hypothyroidism; PAX: Paired box.
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**Conflicts of interest**

None.

**References**

1. Rastogi MV, LaFranchi SH. Congenital hypothyroidism. Orphanet J Rare Dis 2010;5:17. doi: 10.1186/1750-1172-5-17.

2. Zampronii I, Grasberger H, Cortovm F, Vignone MC, Chiumello G, Mora S, et al. Bilateral activation of the dual oxidase maturation factor 2 (DUOX2) gene as a novel cause of congenital hypothyroidism. J Clin Endocrinol Metab 2008;93:605–610. doi: 10.1210/jc.2007-1944.

3. Vilain C, Rydlewski C, Duprez L, Heinrichs C, Abramowicz M, Malvaux P, et al. Autosomal dominant transmission of congenital hypothyroidism due to loss-of-function mutation of PAX8. J Clin Endocrinol Metab 2001;86:234–238. doi: 10.1210/jc.2001.8117140.

4. Vono-Toniole J, Rivolta CM, Targovnik HM, Medeiros-Neto G, Kopp P. Naturally occurring mutation in the thyroglobulin gene. Thyroid 2005;15:1021–1033. doi: 10.1089/thy.2005.15.1021.

5. Dimitri P, Habib AM, Gurbuz F, Millward A, Wallis S, Moussa K, et al. Expanding the clinical spectrum associated with GLIS3 mutations. J Pediatr Endocrinol Metab 2015;28:13–24. doi: 10.1515/jpem-2014-0377.

6. Sun Y, Bak B, Schoenmakers N, van Trotsenburg AS, Oostdijk W, Mora S, et al. Biallelic inactivation of the dual oxidase maturation factor 2 (DUOX2) gene in a cohort of Polish patients with primary congenital hypothyroidism and dysgenetic thyroid glands. J Pediatr Endocrinol Metab 2015;28:735–743. doi: 10.1515/jpem-2014-0310.

7. Doyle DA, Gonzalez I, Thomas B, Scavina M. Autosomal dominant linked syndrome of central hypothyroidism and testicular enlargement. Nat Genet 2012;44:1375–1379. doi: 10.1038/ng.2380.

8. Thorwarth A, Schnittert-Hubener S, Schrumpf P, Muller I, Jyrch S, Dame C, et al. Comprehensive genotyping and clinical characterisation reveal 27 novel NKX2-1 mutations and expand the phenotypic spectrum. J Med Genet 2014;51:375–387. doi: 10.1136/jmedgenet-2013-102248.

9. Vhet GV. Development of the thyroid gland: lessons from congenital hypothyroidism mice and men. Clin Genet 2003;63:445–455. doi: 10.1111/j.1399-0004.2003.00107.x.

10. Al Taji E, Biebermann H, Limanova Z, Hnikova O, Zikmund J, Kopp P, et al. Naturally occurring mutation in the thyroglobulin gene. Thyroid 2005;15:1021–1033. doi: 10.1089/thy.2005.15.1021.

11. Al Taji E, Biebermann H, Limanova Z, Hnikova O, Zikmund J, Kopp P, et al. Expanding the clinical spectrum associated with GLIS3 mutations. J Pediatr Endocrinol Metab 2015;28:13–24. doi: 10.1515/jpem-2014-0377.

12. Sun Y, Bak B, Schoenmakers N, van Trotsenburg AS, Oostdijk W, Mora S, et al. Biallelic inactivation of the dual oxidase maturation factor 2 (DUOX2) gene in a cohort of Polish patients with primary congenital hypothyroidism and dysgenetic thyroid glands. J Pediatr Endocrinol Metab 2015;28:735–743. doi: 10.1515/jpem-2014-0310.

13. Liu S, Wang X, Zou H, Ge Y, Wang F, Wang Y, et al. Identification and characterization of novel PAX8 mutations in congenital hypothyroidism (CH) in a Chinese population. Oncotarget 2017;8:8707–8716. doi: 10.18632/oncotarget.14419.

14. Esperante SA, Rivolta CM, Miravalle V, Iorcansky S, Billel M, et al. Identifying novel variants of four PAX8 exonic and intronic loci in patients with congenital hypothyroidism and dysgenetic thyroid glands. Clin Endocrinol (Oxf) 2008;68:828–835. doi: 10.1111/j.1365-2265.2007.03011.x.

15. Adzhubei I, Jordan DM, Sunyaev SR. Predicting functional effect of human missense mutations using PolyPhen-2. Curr Protoc Hum Genet 2013;Chapter 7:20. doi: 10.1002/0471142953.mh072076.

16. Ng PC, Henikoff S. SIFT: Predicting amino acid changes that affect protein function. Mol Cell Proteomics 2003;3:1382–1384. doi: 10.1074/mcp.M300017-MCP200.