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] 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*), dualoxidase 2 (*DUOX2*),

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DUOXA2, thyroglobulin (*TG*) and *IYD/DEHAL1* [Supplementary Table 1, http://links.lww.com/CM9/A29].

Besides, there also exist rare syndromic diseases with CH, such as Bamforth-Lazarus syndrome, neonatal diabetes mellitus and congenital hypothyroidism (NDH) syndrome,[5] central hypothyroidism and testicular enlargement (CHTE)[6] and choreoathetosis, CH with or without pulmonary dysfunction (CAHTP; also referred to as brain-lung-thyroid syndrome).[7,8] It has been reported that some thyroid transcription factors are essential for thyroid organogenesis, paired box 8 (*PAX8*) is one of these important factors.[9]

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, athyrosis, 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,[10] whereas only 0.5% of patients with CH was reported among Czechs.[11] 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 2.38% was reported in Guangxi,[12] 2.73% in Shanghai,[13] and 1.14% in Shandong province.[14] 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 DELFIA 1235; PerkinElmer, Wellesley, MA, USA). Subjects with increased TSH ($\geq$8 mU/L) levels were recalled for further evaluation.

Serum TSH and free thyroxine (FT4) were determined by electrochemiluminescence assay (Cobas e601; Roche, Basel, Switzerland). Diagnosis of CH was based on elevated TSH level (TSH $\geq$ 10 mU/L) and decreased FT4 level (FT4 < 12 pmol/L). Thyroid ultrasonography and ^{99m}Tc scintigraphy were performed during the neonatal period before treatment. The scintigraphy and ultrasonography were conducted and interpreted by a number of specialized physicians in nuclear medicine and radiology, respectively.

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

Table 1: Samples with rare variants in the known causal genes of CH.

Samples	Genes with rare variants			
	PAX8	*TG*	*TPO*	*DUOX2*
1	rs189229644	/	/	/
2	rs189229644	rs74590117	rs114406277	rs144153950
3	c.275T>C	rs10091530	/	/
4	c.398G>A	/	/	/

CH: Congenital hypothyroidism; *DUOX2*: Dualoxidase 2; *PAX8*: Paired box 8; *TG*: Thyroglobulin; *TPO*: Thyroid peroxidase.

sequencing were confirmed by MassArray iPLEX platform (Agena Bioscience, San Diego, CA, USA) with Agena Bioscience's complete iPLEX Gold Genotyping Reagent Set 384 (Lot No.: 10148-2) according to the manufacturer's instruction at Hebei Jianhai Biochip Technology Co., Ltd. (China).

Polyphen-2[15] and Sorting Intolerant From Tolerant (SIFT)[16] were used to evaluate the pathogenicity of the identified mutations. Proteins sequences of different species were downloaded from NCBI Genbank and aligned with CLUSTALW.

Constructs preparation

The 499 bp promoter region of human *TPO* gene (chr2: 1,416,733–1,417,232) were amplified by polymerase chain reaction using primers 5′-ATctcgagAGAAAAC-TAGGGGCTTGGGG-3′ and 5′-ATaagcttATAAACT-GACTCCGCGCTTG-3′ by FastPfu DNA Polymerase (Transgen, Beijing, China). The PCR products and empty pGL3-basic were then double digested by *Xho*I and *Hind*III in NEBuffer 2.1 (NEB, Ipswich, MA, USA) at 37°C for 6 h. The digested PCR product and pGL3-basic were purified and ligated together with T4 DNA ligase (NEB).

The opening reading frame (ORF) of human *PAX8* (NM_003466) was synthesized and cloned into pcDNA3.1(+) vector at a commercial biotechnology company (Tsingke, Beijing, China). The two mutations were also introduced into wild type *PAX8* by synthesis.

Cell culture and luciferase assay

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% CO_2. When cells confluency reached 90%, transfection was performed using Lipofectamine 2000 (Thermo Fisher) for wild type or mutant *PAX8* plasmids together with pGL3-P_{TPO} 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) only. 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

Table 2: Annotations of variants for the four CH samples with *PAX8* variants.

				Minor allele frequencies			Predictions	
Genes	Variants	mRNA	Protein	1000 genome	ExAC	GO-ESP	Polyphen	SIFT
TG	rs74590117	c.4493C>T	p.Thr1498Met	0.0635	0.0044	0.0002	0.810 Deleterious	0.180 Benign
TG	rs10091530	c.7847A>T	p.Asn2616Ile	0	0.0266	0.1146	0.200 Benign	0.020 Deleterious
TPO	rs114406277	c.2305C>T	p.Arg769Trp	0.0050	0.0021	0.0092	0.922 Deleterious	0.180 Benign
DUOX2	rs144153950	c.2894C>T	p.Ser965Leu	0	0.0001	0.0002	0.064 Benign	0.490 Benign
PAX8	rs189229644	c.806C>T	p.Pro269Leu	0.0040	0.0007	0	0.002 Benign	0.350 Deleterious
PAX8	Novel	c.275T>C	p.Ile92Thr	/	/	/	1.000 Deleterious	0.000 Deleterious
PAX8	Novel	c.398G>A	p.Arg133Gln	/	/	/	0.999 Deleterious	0.000 Deleterious

CH: Congenital hypothyroidism; *DUOX2*: Dualoxidase 2; ExAC: A large-scale Exome sequencing projects for 60,706 unrelated individuals by the Exome Aggregation Consortium; GO-ESP: GO Exome Sequencing Project for 6503 unrelated individuals by the National Heart, Lung, and Blood Institute; PAX8: Paired box 8; SIFT: Sorting Intolerant From Tolerant; TG: Thyroglobulin; TPO: Thyroid peroxidase.

Figure 1: Distribution of known pathogenic mutations of *PAX8*. PAX: Paired box.

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 synthesized in pcDNA3.1(+). The two mutations of *PAX8* (Ile92Thr and Arg133Gln) were 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 HEK293 cells were transfected. 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.

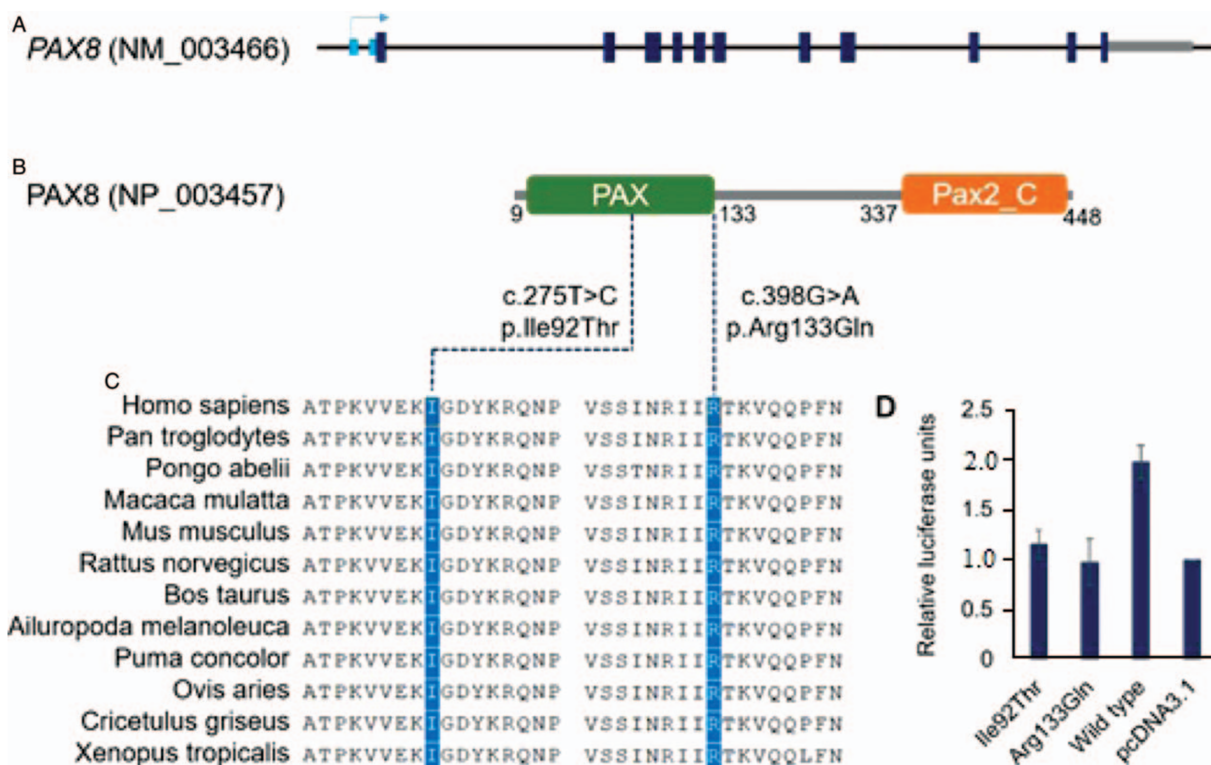

Figure 2: Characterization of *PAX8* and two mutations in our cohort of CH samples. (A) Diagram of *PAX8* gene. Arrow indicates the transcription direction. (B) Diagram of *PAX8* protein. (C) Multiple sequence alignment of *PAX8* from 12 different species. (D) Luciferase reporter assay of the two mutations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

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.[3] 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.[10,12-14,17-32] Although inherited through autosomal dominant transmission, the penetrance varied greatly for some *PAX8* mutations.[3] It is worth noting that the clinical phenotypes of individuals with heterozygous *PAX8* mutations vary considerably within the same family.[3]

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.[20,21] 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.[11,18] 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%[22] in Shandong to 2.73% in Shanghai.[13]

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.

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Conflicts of interest

None.

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