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Genetic testing of *PAX8* mutations associated with thyroid dysgenesis in Chinese congenital hypothyroidism patients

Miaomiao Li*[1, 2]*, Fang Wang*[3]*, Xiuli Wang*[4]*, Yucui Zang*[1, 2]*, Wenmiao Liu*[1, 2]*, Fengqi Wang*[1, 2]*, Lu Zhang*[1, 2]*,
***Qian Tang*[1, 2]*, Shiguo Liu*[1, 2]*, Dehua Zhao*[5]**

[1]*Medical Genetic Department, The Affiliated Hospital of Qingdao University, Qingdao, China*
[2]*Prenatal Diagnosis Center, The Affiliated Hospital of Qingdao University, Qingdao, China*
[3]*Department of Endocrinology and Metabolism, The Affiliated Hospital of Qingdao University, Qingdao, China*
[4]*Neonatal Disease Screening Center, Xuzhou Maternity and Child Health Care Hospital, Xuzhou, China*
[5]*Department of Henan Newborn Screening Center, The Third Affiliated Hospital of Zhengzhou University, Zhengzhou, China*

Abstract

Introduction: Thyroid dysgenesis (TD) is the main cause of congenital hypothyroidism (CH), affecting nearly 1 in 2000–3000 newborns worldwide, as the most common neonatal endocrine disorder. Paired box gene 8 (*PAX8*), expressed during all stages of thyroid follicular cell, plays a key role in thyroid morphogenesis by a complex regulatory network. In conclusion, the genetic mechanism of *PAX8* mutant in TD is still ambiguous; therefore, further research is needed.

Material and methods: Blood samples were collected from 289 TD patients in Shandong Province, China. Genomic DNA was extracted from peripheral blood. All the exons of *PAX8* along with their exon-intro boundaries were amplified by PCR and analysed by Sanger sequencing.

Results: We identified three novel *PAX8* nonsense mutations in three patients by sequence analysis of *PAX8*: Patient 1 (c.285C>G, p.Tyr95Ter), Patient 2 (c.747T>G, p.Tyr249Ter), and Patient 3 (c.786C>A, p.Tyr262Ter). All the three patients carrying *PAX8* variants had obvious clinical phenotypes of thyroid anomaly, such as hypoplasia and athyreosis.

Conclusion: We conducted the largest worldwide *PAX8* mutation screening so far in TD patients. Three presumably pathogenic *PAX8* mutations were detected in 289 TD cases for the first time, showing the mutation rate of *PAX8* is 1.04% in Chinese TD patients. In addition, our study expands the gene mutation spectrum of TD. **(Endokrynol Pol 2020; 71 (2): 153–159)**

Key words: *thyroid dysgenesis; paired box gene 8; mutation; Sanger sequencing*

Introduction

Congenital hypothyroidism (CH) is the most common neonatal endocrine disorder affecting nearly 1 in 2000–3000 newborns worldwide, and the severe deficiency of thyroid hormone can lead to mental retardation and growth failure if not treated in a timely manner [1]. Thyroid dysgenesis (TD), the main cause of CH, accounting for 80–85% of CH cases, caused by the abnormalities of thyroid gland development and migration, can be divided into three subtypes (agenesis, ectopy, and hypoplasia) according to the morphology and location of the thyroid gland [2].

In humans, thyroid development can be divided into six stages: the thyroid anlage assembled by thyroid progenitors (E20–22); the appearance of the thyroid bud (E24); the migration of the thyroid (E30–40); the completion of thyroid migration (E45–50); thyroid bilobation and folliculogenesis (E60); and the completion of differentiation and organogenesis (E70) [3]. In this process, thyroid morphogenesis is a coordinated spatial and temporal process, which, when altered, can result in agenesis, ectopy, and hypoplasia [4, 5]. Various transcription factors play important roles in the thyroid development, especially haematopoietically expressed homeobox gene (*HHEX*), thyroid transcription factor 1 (*TTF1*/*NKX2.1*), thyroid transcription factor 2 (*TTF2*/*FOXE1*), and paired box gene 8 (*PAX8*), the expression of which can be detected at E20 and forms a complex regulatory network to induce morphological changes [6]. *PAX8* regulates the expression of *FOXE1*, *HHEX*, *DUOX2*, *TG*, and *TPO* [7–9] but can be regulated by *HHEX* and *NKX2.1* simultaneously. In addition, *PAX8* expression is autoregulated; the cross-regulatory network ensures that *PAX8* is a master regulator in thyroid development. Furthermore, mutations in *PAX8* combined with *NKX2.1*, *FOXE1*, *NKX2.5*, *TSHR*, *NTN1*, *JAG1*, *BOREALIN*, and *GLIS3* have been identified in patients with TD [10].

PAX8 (NM_003466.4), located on human chromosome 2q12-q14, can be divided into 12 exons. The PAX8 protein has a bipartite functionality consisting of a highly conserved DNA binding region in N-terminal

Shiguo Liu, The Affiliated Hospital of Qingdao University, No. 16 Jiangsu Road, 266000 Qingdao, China; e-mail: liushiguo2002@126.com;
Dehua Zhao; Department of Henan Newborn Screening Center, The Third Affiliated Hospital of Zhengzhou University, Zhengzhou, China; e-mail: zhaodehua369@163.com

and a transactivation region in C-terminal [11, 12]. Expressed during all stages of thyroid follicular cell (TFC) and in adults [13], *PAX8* plays a key role in thyroid morphogenesis. In *pax8*$^{-/-}$ mice at E11.5, thyroid primordium appears to be much smaller (hypoplastic thyroid) than in wild-type and is essentially undetectable at E12.5 follicular cells [14, 15]. In vitro, *PAX8* is a master gene for the regulation of the thyroid differentiated phenotype in several thyroid-derived cell lines [16]. Therefore, *PAX8* is required for thyroid bud survival and TFC differentiation, and mutations in *PAX8* may lead to TD [16, 17]. In the present study, we aimed to identify potential pathogenic *PAX8* mutations in 289 Chinese children with TD, thereby providing insights into its aetiology.

Material and methods

Patients

Sixty-three TD patients were collected for screening variations in exon3 and exon4 of *PAX8* in our preliminary study. In this research, we collected another 289 patients with TD identified through screening of newborns in Shandong Province from January 2015 to November 2017. Neonatal screening for CH was proceeded in all of the subjects 72 hours after birth with blood samples from the heel. Then the concentrations of thyroid-stimulating hormone (TSH), free/total triiodothyronine (T3), and free/total thyroxine (T4) in serum were detected, respectively, using electro-chemiluminescence kits: Elecsys TSH, Elecsys FT3III, and Elecsys FT4III (Roche, German). The diagnosis of CH was based on a high serum TSH level (TSH ≥ 10 mIU/L) and a low fT4 level (fT4 < 12 pmol/L). When the CH patients were three years old, they underwent thyroid echography and scintigraphy to establish the cause of CH. All the 289 patients selected for further research had been diagnosed as TD. The present study was approved by the Ethics Committee of the Affiliated Hospital of Qingdao University (2013-qdfy22). Informed consent was obtained from all individuals included in this study. The research related to human use complied with all the relevant national regulations and institutional policies, was in accordance the tenets of the Helsinki Declaration, and was approved by the authors' Institutional Review Board or equivalent committee.

Methods

Genomic DNA was extracted from peripheral blood with TIANGEN blood kit (TIANGEN, Beijing, China). All the exons of *PAX8* along with their exon-intro boundaries were amplified by PCR, with the specific primer as Table 1. The PCR reaction solution contained

Table 1. ***The primer sequence for polymerase chain reaction (PCR) of PAX8***

Primer	Sequence	Tm	PCR product	Product length [bp]
E1-F	AGGGCATCCTACAGAGACCA	55	Exon 1	547
E1-R	TCCCGTTTAACTTGGGAGGG	56		
E2-F	TCCTCCTACTCCTGGCAGAC	60	Exon 2	471
E2-R	AGAGATCCCCTCACCGATCC	60		
E3-F	TTGGGAGTGAGAACTGGGGA	60	Exon 3	421
E3-R	GGGGAATTCTCTAGCTGCCC	60		
E4-F	GAGGCCTTTAGCAGAGGGTG	60	Exon 4	451
E4-R	GACACCAGAGGCTGCTTTCT	60		
E5-F	GGGTGTCAAAAAGGCGACTG	60	Exon 5	372
E5-R	TCAGTGAATCTGCCCTGGGA	60		
E6-F	ACTCTCACTCCCTGACCCTC	60	Exon 6	446
E6-R	CACATGCAGAGCCCCTACAA	60		
E7-F	GCCCTTTTTCTCCCTCCACA	60	Exon 7	549
E7-R	ATCATCAGGTTGTGCTGCCA	60		
E8-F	TGCCGAGTGGAGTTGAGAAC	60	Exon 8	414
E8-R	CTGGGCCCACCTGGC	59		
E9-F	CTTGGCTTGTGCGTGTTCC	60	Exon 9	401
E9-R	CTCCAAAAGTTGCCGGAGGA	60		
E10-F	GTGGGAATGGCATGGAGGAA	60	Exon 10	468
E10-R	GTCTCAGCCCCTCCCTTTTC	60		
E11-F	CTCCAACTGTCTCCCCAACC	60	Exon 11	452
E11-R	CATGGGCTTGAGAAGCAGGA	60		
E12-F-1	CAGGGAAGGCTATGGTGCAA	60	Exon 12-1	1508
E12-R-1	GTGAGGTACCCAGCGTTCAA	60		
E12-F-2	CATCAGAGCTGAGTAGCCGA	59	Exon 12-2	1389
E12-R-2	ACAGTCAACAAACACCCGCT	60		

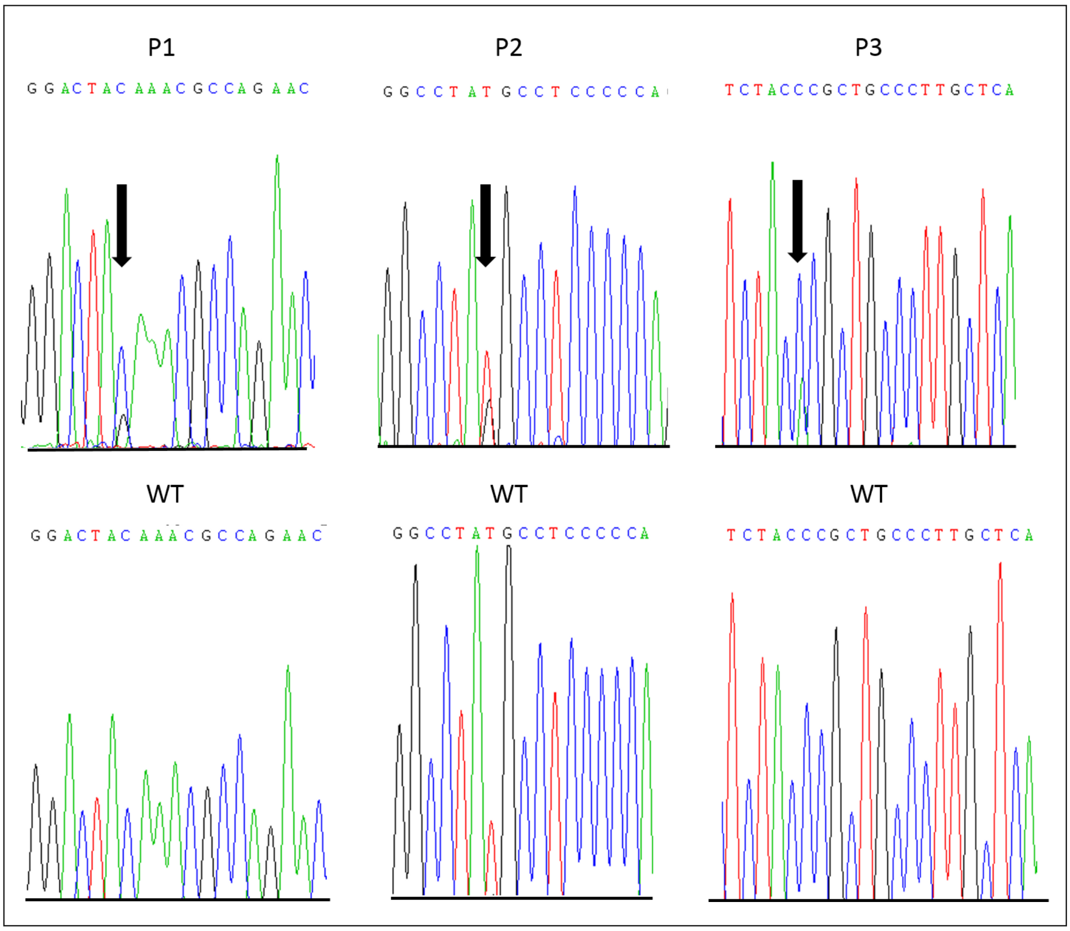

Figure 1. *Sequence maps of PAX8 gene. P1 — sequence of Patient 1 with the PAX8 variant c.C285G; P2 — sequence of Patient 2 with the PAX8 variant c. T747G; P3 — sequence of Patient 3 with the variant c. C786A; C1, C2, and C3 is the corresponding sequence in the general population*

1 × TransStart® FastPfu buffer with 0.2 mM dNTP, 1.25 units of TransStart® FastPfu DNA polymerase, 50 ng Genomic DNA, and 0.2 μM of each primer; the total volume was 25 μl. The procedure of the PCR amplification was as follows: step 1 — denaturation at 95° for 2 min; step 2 — denaturation at 95° for 20 s; step 3 — annealing at primer-specific temperatures for 20 s; step 4 — extension at 72° for 20 s or 60 s; step 5 — incubated at 72° for 5 min. Steps 2 to 4 were cycled 35 times. A BigDye® Terminator Cycle Sequencing Kit and automated sequencer ABI 3730XL were used for the sequencing reaction of the PCR products. The sequencing results were interpreted using BioEdit software.

Results and clinical report

Genetic screening of PAX8 mutation

A total of 289 TD patients were enrolled in this study, the ratio of male to female was 1:1.05. According to the location and size of the thyroid gland, TD was classified into agenesis (120 cases, 41.5%), ectopy (94 cases, 32.5%), and hypoplasia (75 cases, 26%). Sanger sequencing analysis of PAX8 leading to the discovery of three novel *PAX8* variants in three patients: Patient 1 (NG_012384.1 (NM_003466.4): c.285C>G, p.Tyr95Ter); Patient 2 (NG_012384.1 (NM_003466.4): c.747T>G, p.Tyr249Ter), and Patient 3 (NG_012384.1 (NM_003466.4): c.786C>A, p.Tyr262Ter); the sequence maps of the variants is shown in Figure 1. All the variants located in the evolutionary conserved protein domains of PAX8 were not detected in 200 control individuals or in the Exome Sequencing Project (ESP) or the 1000 Genomes Project databases.

Analysis of the relationship between genotype and phenotype

The three patients carrying *PAX8* mutations had obvious clinical phenotype of thyroid anomaly, such as hypoplasia and athyreosis (Tab. 2). The medical records in detail are as follows.

Patient 1, a female infant with p.Tyr95Ter mutation in *PAX8*, was born at 39 weeks of gestation by vaginal delivery with 3250 g birth weight. High TSH levels (208 μIU/mL) were detected at five days of age during neonatal screening; she was recalled at 18 days of age for further evaluation, and the TSH serum level had increased to 384 μIU/mL, the FT3 level was 2.28 pmol/L, and the FT4 level was 4.12 pmol/L. There was no fam-

Table 2. *Clinical characteristics of four congenital hypothyroidism (CH) patients carrying genetic variants*

Subject	Age	Sex	TSH [uIU/mL]	FT4 [pmol/L]	Variant	Clinical phenotype
Patient 1	10	Girl	384	4.12	p.Tyr95Ter	Hypoplasia
Patient 2	12	Girl	284	8.71	p.Tyr249Ter	Hypoplasia
Patient 3	13	Boy	294	2.8	p.Tyr262Ter	Athyreosis

TSH — thyroid-stimulating hormone; FT4 — free thyroxine

ily history of thyroid disease. Tc-99 m scans confirmed hypoplasia. Levothyroxine (L–T4) replacement therapy was started at an initial dose of 25 μg per day. We lost contact with this patient until 3.5 years of age. Then we contacted her parents and learned that the patient was receiving L–T4 33.3 μg replacement therapy. Half a year after withdrawal of L–T4 therapy, her TSH levels were outside the normal range for her age (five years). Therefore, L–T4 25 μg replacement therapy has been needed until now.

Patient 2 with p.Tyr249Ter mutation was a female who weighed 3000 g at birth by vaginal delivery. She was recalled for further analysis after high TSH levels (232 μIU/mL) were detected at six days of age during neonatal screening. At 13 days of age, TSH levels were 284 uIU/L, FT3 levels were 4.31 pmol/l, and FT4 levels were 8.71 pmol/L. Therefore, L–T4 25 μg replacement therapy was started immediately with re-examination of TSH levels per mouth. At two years old, she was diagnosed with permanent CH, and persistent treatment was prescribed because TSH levels were outside the normal range after a four-week withdrawal of L–T4 therapy. Tc-99 m scans showed hypoplasia. L–T4 30 ug replacement therapy was restarted. She is now 12 years old, and her physical and intellectual development are normal. The dosage of L–T4 was increased to 62.5 μg per day.

Patient 3 was a male subject with a p.Tyr262Ter mutation. He was born at full-term by caesarean delivery, and his birth weight was 3750 g. Routine neonatal screening showed a high TSH level of 186 μIU/mL at three days of age. Then, the patient was recalled at 19 days to review the serum TSH level which had increased to 294 μIU/mL but the FT4 (2.8 pmol/L) and FT3 (2.2 pmol/L) levels were both low. L–T4 replacement therapy was started immediately at a dose of 25 μg. Tc-99 m scans detected an athyreosis. At two years of age, he was diagnosed with permanent CH. Now he is four years old, with normal physical and mental development.

Discussion

PAX8 induces thyroid morphogenesis by cooperating with other transcription factors, such as HHEX, NKX2.1, and FOXE1. The regulatory function of PAX8 is closely related to its molecular structure, which consists of two functional domains: a paired box domain for DNA binding; and an octapeptide and a residual paired type homeodomain for transaction. The paired box domain consists of 128 amino acids positioned between 9 and 137, the octapeptide is between 180 and 187, and the residual paired type homeodomain is between 228 and 250, all the domains are highly conserved in human PAX protein family [12, 18].

The first description about *PAX8* variants was conducted by Macchia in 1998; three mutations in two sporadic patients (p.R31H, p.L62R) and one familial case (p.R108X) resulted in severe reduction of the DNA-binding activity of *PAX8*, causing thyroid hypoplasia [19]. Vilain identified p.C57Y in a TD patient; the mutation resulted in loss of the ability to activate thyroid peroxidase (*TPO*) gene [20]. In these cases, *PAX8* mutations were inherited in an autosomal dominant manner. However, the same mutation site in a familial case may result in different clinical phenotypes. Esperante described a thyroid hypoplasia patient and his family carrying mutation p.T225M, while the father, brother, and sister were asymptomatic; and a thyroid agenesis patient and her mother carrying mutation p.G336S, while the mother was unaffected, suggesting that the variable penetrance or expressivity of the mutational carrier can be modulated not only by genetic but also by epigenetic factors [18]. In conclusion, the genetic mechanism of *PAX8* mutant in TD is still ambiguous; therefore, more research is needed in future studies.

In present study, all the patients carrying the novel variant of *PAX8* had symptoms of obvious abnormal thyroid. Variant Y95X located at paired box domain, variant Y249X at homodomain, and variant Y262X at transactivation domain of PAX8 protein (Figure 2) led to PAX8 dysfunction, with most or all of transactivation domain lost. Carrying the heterozygous variant, P1-3 was detected with high level of TSH during neonatal screening, and then P1 and P2 were diagnosed as hypoplasia by ultrasound examination; P3 was athyreosis. It is possible that the nonsense variants led to nonsense mediated decay of the mutated mRNA, thus the TD phenotype could be due to haploinsufficiency of PAX8 protein. The actual functional consequences of *PAX8*

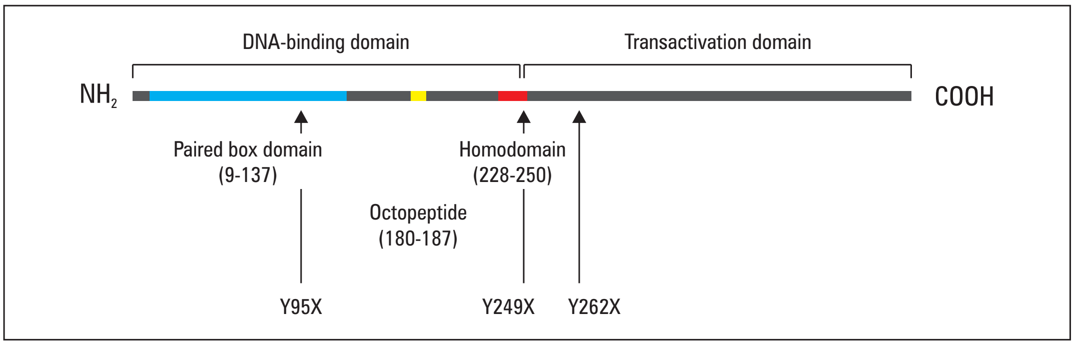

Figure 2. *Schematic representation of human PAX8 protein domains*

truncating mutations are yet to be further investigated, and thus more experiments in vitro are still needed for pathologic study.

Because *PAX8* plays a key role in thyroid morphogenesis, many researches have screened *PAX8* mutations in a large number of CH patients to get the mutational frequencies and relationship between genotypes and phenotypes. Kumorowicz-Czoch found two novel heterozygous substitutions (c.68G>A, p.G23D; c.*416C > T) in 48 Polish CH patients, and the *PAX8* mutation rate is 4.17% [21], while Al Taji E identified a novel mutation (c.155G>C, p.R52P) in 170 CH patients, and the *PAX8* mutation rate in the Czech Republic is 0.6% [22]. In addition, Ramos HE analysed 35 patients with thyroid hypoplasia in southern Brazil and identified a patient with *PAX8* mutation (c.155G>C; p.R52P), and suggested the mutation rate to be 2.9% [23]. Cangul and Kirsten Lanzerath did not find any *PAX8* mutation in 120 CH patients in Pakistan and the United Kingdom and 95 CH patients in south-west Germany, respectively [24, 25], showing the low mutation rate of *PAX8* in these countries. All these findings confirmed the contribution of *PAX8* mutations to the aetiology of CH with a variable penetrance, and rare overall incidence.

In 2012, we analysed exon3 and exon4 of *PAX8* in 300 CH patients, and then reported a heterozygous missense mutation (c.G92>A, p.R31H) and a variation (c.122G>T, p.G41V) in PAX8, showing that the PAX8 mutation rate (0.67%) is very low in CH patients in China [26]. In 2015, we collected 63 TD patients and found a heterozygous missense de novo mutation (c.155G>C, p.R52P) in *PAX8* by sequencing exon3 and exon4; the mutation rate in Chinese TD patients is 1.59% [27]. The mutation rate of *PAX8* in TD patients is obviously higher in CH patients, illustrating *PAX8* induced CH by influencing thyroid development or migration from a different aspect. To further determine the mutational frequencies of *PAX8* in Chinese TD patients, we expanded the sample size to 289 and analysed all the 12 exons and exon-intro boundaries. Ultimately, we discovered three novel variants; the mutation rate was 1.03%.

Conclusion

We conducted the largest worldwide *PAX8* mutation screening so far in TD patients, and three novel *PAX8* nonsense variants were identified in three of 289 TD cases; the mutation rate of *PAX8* was 1.03%. However, there are still two limitations in this study: first, we did not construct the three variants for functional verification; and second, we did not make the genetic analysis in familial cases due to lack of samples from their parents. Therefore, it is necessary to explore the mechanism for the effects of mutations and screen the mutations of *PAX8* among large samples in future research.

Acknowledgment

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Conflict of interest

The authors declare no potential conflict of interest

Data accessibility

The data used to support the findings of this study are included within the article.

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