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Original Article

A De novo PAX8 mutation in a Chinese child with congenital thyroid dysgenesis

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Abstract: Background: Thyroid dysgenesis (TD) is the most frequent cause of congenital hypothyroidism (CH), but its pathogenesis remains unclear. As a thyroid transcription factor, paired box transcription factor 8 (*PAX8*) is essential for thyroid organogenesis and development. Aim: To screen *PAX8* mutations and characterize the features of these mutations in Chinese TD patients. Materials and methods: Blood samples were collected from 63 TD patients in Shandong Province, China, and genomic DNA was extracted from peripheral blood leukocytes. Exon 3~4 of *PAX8* were analyzed by PCR and direct sequencing. Results: Direct sequencing of *PAX8* revealed a heterozygous missense mutation (c.155G/C, P.Arg52Pro) in one child with agenesis. Genetic screening of the child's family revealed that the clinically unaffected parents do not carry the mutation, suggesting that the identified sequence change is a de novo mutation. Conclusion: We report a heterozygous missense de novo mutation in *PAX8* in one out of 63 unrelated Chinese TD patients, showing that the *PAX8* mutation rate is very low in TD patients in China. However, de novo mutation and epigenetic mechanisms need to be considered in the future study.

Keywords: Congenital hypothyroidism, *PAX8*, thyroid dysgenesis, de novo mutation

Introduction

Congenital hypothyroidism (CH) is the most frequent neonatal endocrine disease, which occurs in about 1:2000 to 1:4000 newborns [1]. Except for central hypothyroidism due to hypothalamic or pituitary defects, CH is characterized by high thyrotropin (TSH) levels reflecting the degree of thyroid hormone deficiency by the missing negative feedback on the pituitary and the hypothalamus. CH can be caused by thyroid dysgenesis (TD; OMIM 218700) which is a consequence of abnormal thyroid gland organogenesis or by thyroid dyshormonogenesis (TDHG; OMIM 274400-274900) when any step of thyroid hormone biosynthesis can be affected. TDHG which has been recognized as a genetic disorder mostly with autosomal recessive inheritance, occurs in nearly 15% of CH cases. While about 85% of CH cases are secondary to TD, which leads to complete absence of the thyroid gland (athyreosis, 35-40%), a normally located but too small thyroid (hypoplasia, 5%), and an abnormally located thyroid gland (ectopy, 30-45%) [1, 2]. Many researchers suggest that thyroid transcription factors are essential for thyroid organogenesis, such as paired box transcription factor 8 (*PAX8*), thyroid transcription factor 1 (*TTF1*), and thyroid transcription factor 2 (*TTF2*) [3].

The human *PAX8* which contains a 4-kbp coding sequence divided into 12 exons, is located on chromosome 2q12. As a member of the paired homeodomain transcription factor family, PAX8 contains a paired box domain at the amino terminal end, a transcription activation domain at the carboxy terminal and a paired-type homeodomain in the centre [4]. PAX8 performs key functions not only in thyroid follicular cell differentiation during early embryonic days [5], but also in the maintenance of thyroid follicular cells during all stages of development and in adulthood when PAX8 is expressed as a

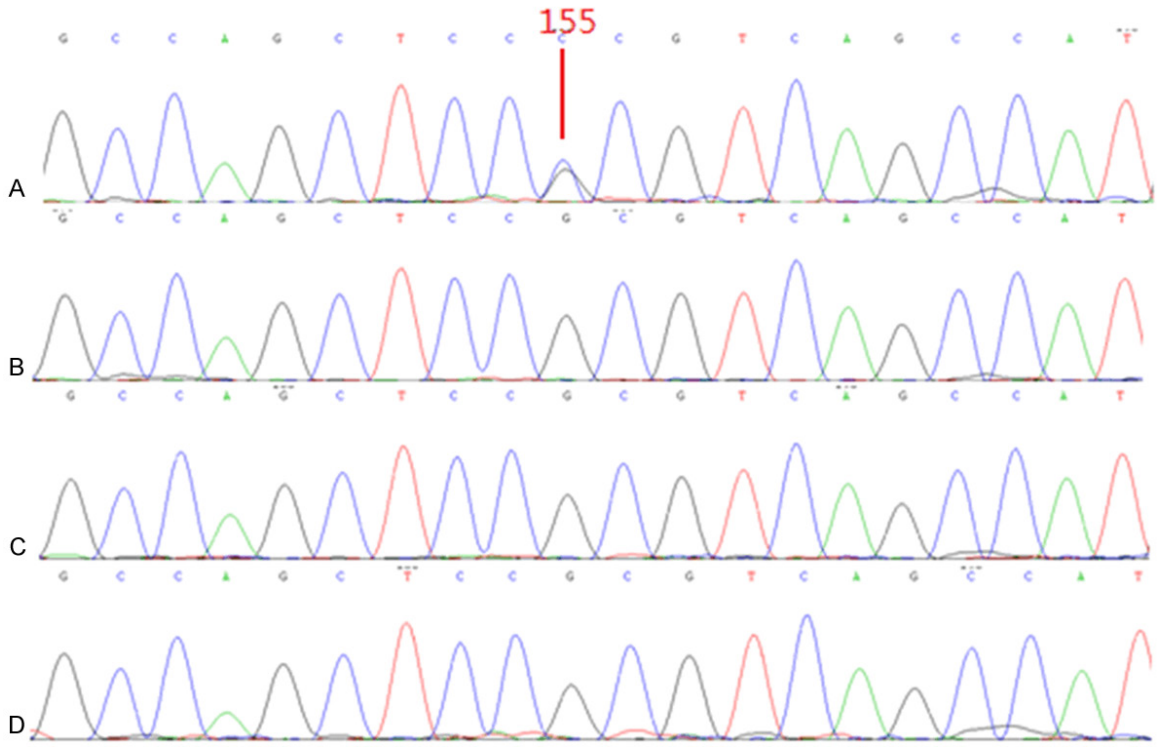

Figure 1. Partial sequences of exon 3 in PAX8 from proband, his parents and a normal control are shown. A: The proband with mutant-type (the heterozygous G and C at nucleotide 155); B: (the father); C: (the mother); D: (a normal control): they are all wild-type (the homozygous G at nucleotide 155).

transcription factor of the thyroperoxidase (*TPO*), thyroglobulin (*TG*) and sodium/iodide symporter (*NIS*) genes by binding to their promoter regions [6].

So far, 15 different *PAX8* mutations have been identified in cases of CH [7]. Moreover, most of these mutations are located in exons 3-4 of *PAX8*. Here, we screened *PAX8* mutations to characterize the features of these mutations and reported the discovery of the first de novo *PAX8* mutation in a Chinese child with TD.

Materials and methods

Patients

A total of 63 TD unrelated patients (19 boys, 44 girls, sex ratio 1:2.3, age 1.8 ± 0.6 years), which were examined to make sure that they did not have other congenital anomalies, were recruited through the neonatal screening program in Jinan, Shandong Province, China, from 2008 to 2012. According to the same test standard, all measurements were done using the same assay. Neonatal screening for CH using filter paper was conducted in all of the subjects at 72 hours after birth, blood samples were collected from the heel and TSH level was measured by enzyme-linked immunosorbent assay (ELISA). We recalled subjects with increased TSH (TSH ≥ 20 μIU/ml) levels observed during neonatal screening for further evaluation. Using electrochemiluminescence assay serum TSH (normal range 0.27-4.2 μIU/ml) and free thyroxin (FT4, normal range 12-22 pmol/L) were determined. CH is diagnosed with high serum TSH level and low level of FT4. Using thyroid scintiscan or thyroid ultrasound examinations, all patients were diagnosed as TD. TD cases were divided into four groups according to thyroid location and size: agenesis (33 cases, 37.8%), ectopy (3 cases, 44.7%), hypoplasia with normal location (27 cases, 17.5%) through ^{99m}Tc thyroid scan or thyroid ultrasound examinations. Additionally, we selected 200 normal control individuals from our sample library. This present study was approved by the Ethics Committee of Jinan Maternity and Child Health Care Hospital and the Affiliated Hospital of Qingdao University. After written informed con-

Figure 2. Comparison of PAX8 protein sequences in the region of the mutation (P.Arg52Pro) from different species indicates that the region is highly conserved.

sent was obtained, we collected blood samples from the children with TD.

DNA analysis

Genomic DNA was extracted from peripheral blood leukocytes by using the QIAamp blood kit (QIAGEN, Hilden, Germany). Two pairs of specific primers which were designed by Primer 5, were used for amplification of the exon 3~4 of PAX8 gene. The primers of exon 3 as follows: forward (5'-TTGGGATTCTCTATT-3') and reverse (5'-TCCTGATTTCCCCAAAG-3'); the primers of exon 4 as follows: forward (5'-CTCTGGCTAAATCCCTGTC-3') and reverse (5'-CTCCCTGCCTGATTGTTC-3'). PCR was performed in 25 μL, using 250 nM dNTPs, 100 ng of template DNA, 0.5 μM of each forward and reverse primer, and 1.25 U AmpliTaq Gold DNA polymerase, in 1 × reaction buffer (10 mM Tris HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$). Samples were denatured at 94°C for 5 minutes followed by 35 cycles of amplification. Each cycle consisted of denaturation at 95°C for 30 seconds, primer specific annealing temperature for 45 seconds, and primer extension at 72°C for 45 seconds. After the last cycle, the samples were incubated for an additional 10 minutes at 4°C to ensure that the final extension step was complete. The amplified products were analyzed in 1.5% agarose gel. In order to perform mutational analysis, amplified PCR products were purified and sequenced using the appropriate PCR primers and the DNA sequencing kit-BigDye Terminator Ready Reaction Cycle Sequencing Kit (PE Applied Biosystems, Warrington, UK) and run on an automated sequencer, ABI 3730XL (Applied Biosystems). The same region was sequenced in 200 blood samples from control individuals.

Results

Genetic analysis

Out of 63 unrelated Chinese TD patients, we just found one missense mutation in one child with TD. The mutation results in nucleotide C to G subsititution in the exon 3 (c.155G/C) (**Figure 1**), leading to an amino acid exchange of arginine to proline at codon 52 (P.Arg52Pro) in the DNA binding domain of PAX8. The PAX8 family protein-sequence of various species including Homo sapiens, Mus musculus, Rattus norvegicus, Danio rerio, Canis lupus familiaris, and Bos taurus was obtained from the NCBI website. Then we carried out multiple sequence alignment of the PAX8 family of different species by DNAMAN software. The mutation was found to be located in the region of PAX8 that was highly conserved between species (**Figure 2**).

Proband parents did not carry this mutation (**Figure 1**), Meanwhile, we also did not find the mutation in 200 control individuals. Additionally, the mutation was not discovered in the normal Chinese population in the database of dbSNP and 1000 genomes. These data suggest that the variant is a de novo mutation.

Clinical features of child with PAX8 De Novo mutation

The index patient was born as the first son and only child of the clinically unaffected parents through vaginal delivery at 40 weeks of gestation in 2007. Birth weight was 3400 g, length was 51 cm and Apgar score was 10. Neonatal screening for CH was positive (TSH spot test, 49.61 mIU/l). At the age of 24 days, the patient was recalled to confirm the test results, which

revealed a high TSH level (105.89 mIU/l, normal range 0.25-5 mIU/l) and a low fT4 and fT3 level (fT4 9.28 pmol/l, normal range 10-26 pmol/l, fT3 3.62 pmol/l, normal range 4.2-8.1 pmol/l). He was diagnosed as CH. At that time, his body weight was 4,200 g and his length was 54 cm. Levothyroxine (L-T4) replacement therapy was started immediately at an initial dose of 25 μg/d. Replacement therapy was modified during follow-up according to clinical and hormonal evaluations to maintain normal serum TSH and FT4 levels. At the age of 30 months, Color Doppler ultrasound examination showed an athyreosis thyroid. Now, at the age of 7 years, he is diagnosed as permanent CH under replacement therapy of 75 μg/d L-T4 and luckily, intellectual and physical development is normal.

Discussion

PAX8, characterized by the presence of a paired domain, is a transcription factor of the mammalian family of PAX proteins [8]. PAX8 is essential for thyroid organogenesis and development. Studies on animal models have demonstrated that *PAX8* is critical for thyroid organogenesis and thyroid cell differentiation. $Pax8^{-/-}$ knockout mice can live at birth, however show growth retardation and short lifespan [5]. In these mice, the thyroid gland is seriously altered and follicles and thyroid follicular cells (TFCs) cannot be found, while parafollicular C cells are present. $Pax8^{-/-}$ knockout mice at E11.5, thyroid primordium appears smaller (hypoplastic thyroid) than in wild-type mice, and at E12.5 follicular cells are essentially undetectable, indicating that Pax8 is required for the survival of thyroid cell precursors [6]. So far, 15 different *PAX8* mutations have been identified in cases of TD. The majority of TD cases is caused by thyroid hypoplasia, however three patients with athyreosis and two with thyroid ectopy have been reported [8]. All cases with TD are heterozygous for the mutations and inherited in an autosomal dominant mode. This displays that in humans, as opposed to mice models, both *PAX8* alleles are necessary for correct thyroid morphogenesis, and a reduced dosage of the gene product (haploinsufficiency) causes dysgenesis.

The molecular mechanisms by which *PAX8* mutations lead to TD are still unclear. Up to now, we just know two different mechanisms. One cause is that affected its transcription activity, another is defects of the DNA-binding property. PAX8-H55Q and S48F mutants located in the paired domain affected the transactivation property of *PAX8* [9, 10]. Furthermore, the PAX8-T277X mutant which causes a premature stop codon after codon 277 is not capable of activating transcription [11]. PAX8 has a paired domain composed of 128 amino acids through which binds to specific DNA sequences and the majority of reported PAX8 mutations are located in this domain. PAX8 performs synergism with TTF1 or TTF2 in activating TPO, TG, NIS transcription by binding to their promoters [12-16]. A S54G heterozygous mutation in *PAX8* was detected in three CH patients with severe hypoplasia in a French family [17] and then functional analysis demonstrates that this mutant decreased DNA binding and transcriptional activity on TG and TPO promoter. So far, five mutations including R31H [18, 19], Q40P [20], S54R [21], L62R [18] and R108X [18] were reported that completely lack or decrease of DNA binding and transcriptional activity on TPO promoter. While six mutations such as p.S48F, p.R52P, p.S54R, p.H55Q, p.K80-A84dup and p.R133Q, were lack of DNA binding or transcriptional activity on TG promoter [9, 10, 21-23].

In this present study, we found a missense mutation (Arg52Pro) in one child with agenesis. This mutation had also been reported in one boy from a Czech three-generation pedigree leading to non-congenital, early-onset hypothyroidism dominantly inherited in the family [22]. The same mutation was detected in his mother and maternal grandmother with early-onset hypothyroidism. For functional characterization, wild-type and mutant proteins were generated using the in vitro cell-free translation, then conducted electrophoretic mobility shift assay (EMSA), binding to an oligonucleotide probe corresponding to the *TG* promoter. The specific complex was identified by competition with unlabelled wild-type proteins. Mutant proteins could not bind to the *TG* promoter element. This showed the R52P mutant results in a loss-of-function effect. Our studies reinforced the pathogenic role of *PAX8* mutation as causal factors in TD.

Interestingly, our proband's clinically unaffected family members were also screened and shown to carry only wild-type sequence of the

PAX8. Our studies suggest this missense sequence mutation identified in the index patient is a de novo mutation. So far, one somatic mutation in *PAX8* (P.K80_A84dup) was detected in the lymphocytes and the tissue with embryonic features but not in the normal tissue and the adenoma of one woman with normal thyroid function and size. However, her children had permanent CH both suffering from the same mutation but inherited by the germline [24]. Therefore, our study is the first time that reported a de novo mutation in an individual with TD. Further arguments against a classic Mendelian inheritance of TD are the high discordance rate of 92% between monozygotic twins, suggesting that beside monogenic, de novo mutation and epigenetic mechanisms could be involved in the pathophysiology of TD [25-27].

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Disclosure of conflict of interest

None.

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