Novel TUBB1 Mutation Related To Thyroid Dysgenesis In 289 Chinese Patients

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Novel *TUBB1* mutation related to thyroid dysgenesis in 289

Chinese patients

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Statement of Ethics: Written informed consent was obtained from the subjects who provided peripheral blood according to the protocol approved by the Affiliated Hospital of Qingdao University.

Impact: 1. Among 289 children with CH and TD, 4 (1.4%) had a c.952C>T(p.R318W) heterozygous mutation in TUBB1, and functional studies indicated that the p.R318W mutant decreased TUBB1 expression and inhibited cell proliferation in a human thyroid cell line. 2. A novel heterozygous missense mutation of TUBB1 in children with CH and TD was identified first in China. 3. The novel finding expands the genotype-phenotype spectrum of TD.

Author Statement: Yangang Wang and Shiguo Liu designed experiments and polished the article. Fang Wang, Chunhui Sun and Miaomiao Li performed the experiments. Fang Wang and Chunhui Sun wrote the manuscript. Yubao Ren and Fengqi Wang analysed the sequencing data and experimental results.

Abstract:

Objective: We aimed to study the types and characteristics of *TUBB1* mutations in a large Chinese cohort with congenital hypothyroidism (CH) and thyroid dysgenesis (TD).

Methods: Mutation of the entire coding region of *TUBB1* was analysed by Sanger sequencing in 289 children with CH and TD from China. Functional studies were further used to identify the effect of novel mutations on thyroid cells.

Results : Among the 289 children with CH and TD, 4 (1.4%) had a c.952C>T(p.R318W) heterozygous mutation in *TUBB1*, resulting in a change from tryptophan to arginine at codon 318 of the TUBB1 protein. Functional studies indicated that the p.R318W mutant decreased TUBB1 expression and inhibited cell proliferation in a human thyroid cell line.

Conclusions : A novel heterozygous missense mutation of *TUBB1* in children with CH and TD was identified first in China, laying the foundations to expand the genotype-phenotype spectrum of TD.

Key words: Congenital hypothyroidism; thyroid dysgenesis; TUBB1; Mutation

Introduction

Congenital hypothyroidism (CH) is subdivided into primary CH and secondary CH^[1]. As one of the most common endocrine diseases, primary CH can lead to cretinism with mental retardation and growth retardation but initial nondescript symptoms partly because of maternal thyroid hormones passing through the placenta^[2]. Therefore, diagnosing and treating CH before clinical manifestation is crucial to improving prognosis based on biochemical measurement during newborn screening^[3] of an elevated serum concentration of isolated thyroid-stimulating hormone (TSH>9 IU/L) and a low free T4 level (FT4<0.6 ng/dL). In secondary or central CH, TSH deficiency occurs because of abnormalities in the structure or function of the pituitary or hypothalamus^[1].

Primary CH, caused by thyroid dysgenesis (TD) or thyroid hormone (TH) synthesis disorders, is the most common form, with an incidence of 1 in 3000-4000 newborns^[4] and a female to male ratio approaching 2:1^[5]. TD accounts for 80-85% of primary CH, including thyroid dysplasia (5%), an ectopic thyroid (30%-45%) and an absent thyroid (35%-45%)^[6]. Thyroid transcription *factor* (*TTF*) candidate genes, such as paired box gene 8 (*PAX8*), NK2 homeobox 1 (*NKX 2.1*), NK2 homeobox 5 (*NKX 2.5*), forkhead Box E1 (*FOXE1*), *NTN1*, *JAG1*, *BOREALIN* and *Glis3*, play indispensable roles in the normal formation and maintenance of thyroid functional differentiation in adults, and previous studies have identified some *TTF* mutation contributing to the incidence of TD^[7-12]. The high prevalence of thyroid developmental anomalies was indicated in first-degree relatives of cases with CH and TD, suggesting that genetic factors are involved in the incidence of TD^[13-15]. To date, only a small fraction of TD mutations have been found, and novel pathogenic genetic mutations must be further identified.

The remaining 15-20% of cases were caused by TH dyshormonogenesis identified by mutations in thyroid peroxidase (TPO), double oxidase 2 (*DUOX2*), dual oxidase activator 2 (*DUOXA2*), Pendrin, and solute carrier family 5 (sodium/iodide cotransporter) member 5 (*SLC5A5*)^[16-19].

In 2018, Athanasia Stoupa *et al.* first reported and further confirmed that *TUBB1* mutations, which can interfere with microtubule assembly and influence thyroid development and function by affecting the proliferation, migration and differentiation of thyroid cells^[20], were associated with CH and TD. *TUBB1* (Tubulin, Beta 1 Class VI) is located at 20q13.32 and contains 5 exons encoding 451 amino acids. Microtubules are assembled by evolutionarily conserved α - and β -tubulin heterodimers as cytoskeletal components of all eukaryotic cells and are involved in cell morphology, motility, differentiation, intracellular transport, signal transduction and mitosis^[21]. Because our previous study revealed that *TUBB1* c.952C>T is largely a pathogenic gene for CH children with TD^[22], we performed exon screening and functional studies verifying *TUBB1* in 289 children with CH and TD in a Chinese population to further demonstrate the pathogenic mechanism of *TUBB1* c.952C>T to expand the pathogenic gene spectrum of CH and TD.

Materials and Methods

Patients

A total of 289 children diagnosed with CH and TD (121 athyreosis, 93 ectopy, and 75 hypogenesis; male: 130, female: 159; average age: 3.1 ± 1.9 years) from Shandong Province, including Jinan, Qingdao, Weifang, Zibo, Linyi, and Liaocheng, were selected by a neonatal screening program between 2007 and 2016. The three criteria for inclusion were as follows: (1) neonatal screening for thyroid-stimulating hormone

(TSH) $>9 \mu\text{IU/mL}^{[1]}$; (2) thyroid ultrasound or thyroid nucleus scan diagnosed as TD; (3) other congenital diseases (blood system, immune system, malignant tumour and mental diseases) other than CH were excluded. This study was approved by the ethics committee of the Affiliated Hospital of Qingdao University (Shandong, China).

Genetic Analysis

DNA samples were extracted from the peripheral blood leukocytes of 289 patients with TD using a QIAamp Blood DNA Mini Kit (QIAGEN Company, America). The primers for *TUBB1* were designed by Primer 5.0. PCR was performed in a total volume of 25 μL using 250 nM dNTPs, 100 ng of template DNA, 0.5 μM of each forwards and reverse primer, and 1.25 U AmpliTaq Gold DNA polymerase in 1 $\times$ reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM MgCl_2). PCR amplifications were subjected to the following reaction conditions: predenaturation at 94 $^{\circ}\text{C}$ for 5 min; 35 cycles of 94 $^{\circ}\text{C}$ for 30 s, 58 $^{\circ}\text{C}$ for 30 s, and 72 $^{\circ}\text{C}$ for 30 s; 72 $^{\circ}\text{C}$ for a final extension of 10 min.

The PCR products were identified by agarose gel electrophoresis and analysed using a BIO RAD Gel DocTM XR+ imaging system. The products appearing as a distinct and single band were Sanger sequenced by Shanghai Sonny Parsonoff Biology Company and compared with the *TUBB1* reference sequence (NM-030773.3) to identify mutations. Biological information analysis of mutations was performed.

Vectors and Plasmids

The phuman *TUBB1*-tagged EGFP vector and control vector pEGFP-C-Flag were purchased from Synbio Technologies (Suzhou, China). Mutant R318 W-*TUBB1* was generated using a PCR-based site-directed mutagenesis method and the Fast Mutagenesis System (Transgen, Beijing). Plasmid DNA was extracted from overnight

cultured cells using an EndoFree Plasmid Mini Kit (CWBIO, Jiangsu) according to the manufacturer's instructions at 37 °C with shaking at 250 rpm.

Cell culture, Cell counting kit-8 and Scratch assays

For the in vitro studies of mutations, Nthy (Nthyori 3.1), an immortal human thyroid cell line, was purchased from Rongbai Biological (Shanghai, China) and cultured in RPMI 1640 medium (BI, Biological Industries) containing L-glutamine supplemented with 10% foetal bovine serum (FBS), 100 IU/mL of penicillin and 100 µg/mL of streptomycin. The Nthy cells were used from passage 4 and were incubated at 37 °C and 5% CO₂ in a humidified atmosphere.

For the CCK-8 assay, Nthy cells were successively digested, counted and plated in 96-well plates (3×10^5 cells per cell) 24 h before transfection and transfected with 100 ng of the TUBB1-tagged EGFP vector, control vector pEGFP-C-Flag and mutant R318 W-TUBB1 once the cells reached 50% confluence using Lipofectamine 3000 (Invitrogen, Thermo Fisher) according to the manufacturer's instructions. After the cells were cultured for 48 h, 72 h, 96 h, or 120 h, 10 µL of WST-8 (Vazyme) was added to the plate, followed by incubation for 2 hours, and then the OD was measured at 450 nm using an enzyme reader (Thermo Fisher Scientific MULTISKAN GO).

The cells were seeded into 6-well plates after digestion and scratched in the middle of each well using an import spear (20 µl) once the cells reached 100% confluence. After washing 3 times with PBS to remove the floating cells, the cells were photographed at 0 h, 10 h, and 24 h using the cellSens Standard system.

RNA isolation, RT-PCR analysis and Western Blotting

Total RNA was extracted from Nthy-ori cells using TRIzol agent (Invitrogen) according to the manufacturer's instructions. A total of 1000 ng of total RNA was mixed with HiScript II qRT SuperMix II (Vazyme Biotech) for complementary DNA synthesis, incubated for 15 min at 50 °C, and then incubated for 5 sec at 85 °C. Each cDNA of RT-PCR was performed using a 20 µL reaction system including 1 µL of cDNA, 0.5 µL of each forward and reverse primer, 10 µL of SYBR PCR master mix (Vazyme) and ddH₂O. The *TUBB1* primer sequences are shown in Table 1. The reactions were incubated in 96-well plates on a real-time PCR system at 95 °C for 2 min and 35 cycles of 95 °C for 5 s and 60 °C for 10 s. GAPDH served as the internal reference using the comparative threshold cycle method ($2^{-\Delta\Delta CT}$) to measure the expression level of the target gene.

After 48 h of transfection, the cells were collected and lysed at a ratio of 100:1 using RIPA and PMSF (Beyotime). The protein concentrations were determined using a BCA kit (Thermo Scientific), and 40 µg was isolated on 12% SDS-PAGE gels. Western blotting was performed according to the routine procedure using mouse monoclonal anti-beta 1 tubulin primary antibody (Sigma) (1:800) and goat anti-mouse secondary antibody combined with horseradish peroxidase (1:5000). The results were obtained using FluorChemQ (ProteinSimple) and the chemiluminescent HRP substrate (Immobilon Western; Millipore).

Statistical Analysis

Statistical analysis was performed using paired Student's t test. $p < 0.05$ represents statistical significance. Graphs were prepared using Graphpad Prism 8.0.

Results

Screening of *TUBB1* mutations in a cohort with CH and TD

Among 289 unrelated patients with CH and TD, we identified six variations in *TUBB1* c.952C>T/R318W, c.658C>T/P220S, c.112G>A/G38R, c.582G>T/E194D , c.997G>A/V333M, and c.1045G>A/V349T. Because the *TUBB1* c.952C>T mutation (Figure 1A) is located on exon 4 and results in the change of tryptophan to arginine at codon 318 of the *TUBB1* protein (p.R318W), it was not detected in 300 normal populations (Figure 1B), and multiple sequence alignment of *TUBB1* from different species (*Homo sapiens*, *Acinonyx jubatus*, *Athene cunicularia*, *Castor canadensis*, *Danio rerio*, *Lagenorhynchus obliquidens*, and *Zonotrichia albicollis*) indicated that R318 was highly conserved (Figure 2), and SIFT and POLYPHEN-2 were used to analyse *TUBB1* c.952C > T. This mutation was evaluated as “potentially pathogenic” based on the classification criteria and guidelines for genetic variation by the American College of Medical Genetics and Genomics^[23] (Table 2).

Clinical data

Patient 1 (P1), P2, P3, and P4, all harbouring the *TUBB1* p.R318W mutation, were born by vaginal delivery after full-term gestation with birth weights of 3100 g, 2800 g, 2900 g, and 3100 g, respectively. P1 and P3 were female while P2 and P4 were male. High TSH levels (141 µIU/mL, 651 µIU/mL, 108 µIU/mL, and 112 µIU/mL) were detected during routine neonatal screening with no family history of thyroid disease. L-T4 replacement therapy was initially administered at a dose of 25 µg, and a follow-up survey was performed. Their TSH levels were 76 µIU/mL, 89 µIU/mL, 67 µIU/mL, and 72 µIU/mL, and ultrasound examination demonstrated thyroid athyreosis at the age of 2 y. They are aged 10 y, 6 y, 8 y, and 4 y now, each with normal physical and intellectual development, except for P2 with underachievement and one standard

deviation behind peers in height. The recommended doses of L-T4 reached 75 µg, 100 µg, 62.5 µg, and 87.5 µg per day.

The R318W mutation reduces the expression of *TUBB1* compared with wild type

Because β 1-tubulin is expressed in the developing thyroid in humans, we examined the mRNA and protein expression in Nthyori 3.1 cells transfected with the TUBB1-tagged EGFP vector (wild type, WT), control vector pEGFP-C-Flag and mutant R318W-TUBB1 to investigate the effect of the mutation. The expression of R318W mutant TUBB1 was significantly decreased compared with that of WT TUBB1 (Figure 3A). Western blotting revealed that the protein expression level of R318W mutant TUBB1 was lower than that of WT TUBB1 (Figure 3B).

R318W mutation inhibits the proliferation of thyrocytes To further examine the effect of the R318W mutation on Nthy cell proliferation, Nthy cells were transfected with 100 ng of the TUBB1-tagged EGFP vector, control vector pEGFP-C-Flag and mutant R318W-TUBB1 for 46 h, 72 h, 96 h, and 120 h, and MST-8 was used to detect the OD 490 value. The R318W mutation inhibited cell proliferation in a time-dependent manner, showing statistical significance compared with the control group at 96 h and 120 h (Figure 4A). Additionally, the cells were seeded into 6-well plates, scratched when the cells reached 100% confluence, and then photographed at 0 h, 10 h, and 24 h using the cellSens Standard system. The R318W mutation did not affect cell migration in Nthy cells (Figure 4B1 and 4B2).

Discussion

The thyroid, derived from a middle anlage of the pharyngeal floor and comprising foregut endoderm cells in the fourth week, is the largest endocrine gland in the human body; the thyroid primordium arrives at its final pretracheal position below the thyroid

cartilage after descending along the midline of the neck^[24-25] during the seventh week of embryonic life. Any factors affecting the development and migration of the thyroid during this period can lead to thyroid dysplasia^[26]. Impaired specification, proliferation and survival of thyroid precursor cells as well as the loss of coordination of movement may lead to TD^[27]. Approximately 50-60% of TD patients have ectopic thyroid glands, usually with a lingual, suprahyoid, or infrahyoid location. Thyroid homeostasis is essential for central nervous system development and physical growth in infants.

TD was previously regarded as a sporadic disease. Leger J *et al.* reported a 7.9% incidence of first-degree relatives in children with TD^[28]. Previous studies have shown that the familial incidence of TD ranges from 2% to 12%, suggesting that TD may be related to genetic factors^[29-30]. Today, more than nine genes have been identified as pathogenic genes in TD, such as *PAX8*, *NKX2.1*, *NKX2.5*, *FOXE1*, *TSHR*, *NTN1*, *JAG1*, *BOREALIN* and *Glis3*. Notably, considering that not more than 5% of patients with TD have shown mutations in these genes^[20], identifying novel pathogenic mutations of TD is crucial.

In 2018, Athanasia Stoupa *et al.* first identified three *TUBB1* mutations, including two missense mutations of c. 479C>T, c. 318C>G, and one frameshift mutation c. 35delG, by targeted second-generation sequencing technology in a cohort of 270 cases with CH and TD. They confirmed that TD was associated with *TUBB1* mutation by abnormal proliferation of early progenitor cells, delayed thyroid migration and impaired thyroid differentiation and thyroid hormone release, and thyroid tissue disorder was found in the *tubb1*^{-/-} mouse model^[20]. *TUBB1* (Tubulin, β 1) is a member of the β -tubulin family of proteins and is expressed in

megakaryocytes and the developing thyroid in humans and mice^[20, 31]. Because microtubules comprise α and β tubulins with highly conserved protein assembly during eukaryotic evolution and multiple genes involved in the coding and expression of tubulin isotypes, the primary sequence of tubulins caused by tubulin mutations would seriously affect the function of microtubules^[32].

Because the prevalence and functional characterization of TUBB1 mutations in a Chinese cohort on CH and TD have not yet been reported and the phenotypic-genotype correlation of TUBB1 remains unclear, our study aimed to further verify the exons of TUBB1 in 289 children with CH and TD, and functional studies were conducted on this mutation based on our previous study. Thus, among the 289 children with CH and TD, 4 (1.4%) cases had a TUBB1 c.952C>T (p.R318W) mutation, resulting in the change of tryptophan to arginine at codon 318 of the TUBB1 protein. Each monomer of tubulin contains three domains comprising 451 amino acids with a molecular weight of 50.3 KDa. The nucleotide-binding domain, comprising residues 1-206, is necessary for microtubule assembly because it plays a role in regulating GTP binding and hydrolysis. Residues 207-384 form the intermediate region of the tubulin sequence as an indispensable catalytic subunit of the molecule, with the function of being involved in hydrolysis and interacting with monomers both along and among protofilaments. The carboxy-terminal domain (residues 385-451) is involved in the assembly of microtubules and interaction with microtubule-associated proteins^[21]. The R318W mutant was located in the intermediate region of the tubulin sequence, showing reduced expression of TUBB1 in thyroid cells expressing the mutant and reduced cell proliferation compared with WT TUBB1 in the CCK-8 assay, suggesting that the mutation is associated with TD via inhibitory effects on cell proliferation and abnormal protein expression. However,

no statistically significant difference was observed in scratch assays between the R318W mutant and WT TUBB1. To date, the molecular mechanism regulating thyroid migration remains unknown^[6, 27]. The migration of wild-type thyroid progenitors is tightly connected by epithelial cadherin with the characteristics of collective migration^[33], which depends on the matrix environment that provides the basis for the directional movement and migration of cells^[34]. No obvious significant difference was found in the migration of WT and mutant thyroid cells in our experiment, likely because of the lack of a matrix environment for cell migration in vitro, and further experiments are needed. The R318W mutant was described by Kunishima et al. in 2009 in patients with macrothrombocytopenia^[35]. However, patients with the R318W mutant in our study cohort had normal platelets, likely because of variable penetrance^[36]. The TSH levels of four patients were all above 100 μ IU/mL by routine neonatal screening, following L-thyroxine therapy until now, with normal physical and intellectual development.

In conclusion, a novel *TUBB1* mutation was found in a cohort study in China on CH and TD, and the mechanism of its pathogenic role in CH and TD was established by functional studies. However, the relationship between *TUBB1* mutation and athyreosis or thyroid ectopia remains unclear and can be confirmed in further detailed studies.

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exon	upstream primer	downstream primer	fragment (bp)	annealing temperature (° C)
1	5' -GAGCTAGGAGCCACAGTCATCA -3'	5' -GTCCTCCCTGCTAACTTAGGGTATTTT -3'	550	58
2	5' -CTTAGTAGAGGCAAAAAGCGAATGA -3'	5' -TGTTAAGGTGTGTGCCATATCCA -3'	500	59
3	5' -CAATATCACAGAGCAGACTGATGGA -3'	5' -TCGGCTCCCTCCGTGTAGT -3'	500	59
4-1	5' -CCTAGAACCTGGGACGATGGA -3'	5' -GTATTGCGGGCATCGAACAT -3'	800	60
4-2	5' -CAAGCTGGCGGTGAACATG -3'	5' -TAGATGTCAGCAAAGACCCTAATGC -3'	800	60

Table 1 Amplification information of exons 1 ~ 4 of *TUBB1*

Figure 1. A. The arrow indicates a CC homozygous genotype at nucleotide 952 of the coding sequence of *TUBB1* in healthy people.

B. The arrow indicates a C/T heterozygous genotype at nucleotide 952 of the coding sequence of the *TUBB1* mutation.

Figure 2. Multiple sequence alignment of the TUBB1 protein from *Homo sapiens*, *Acinonyx jubatus*, *Athene cunicularia*, *Castor canadensis*, *Danio rerio*, *Lagenorhynchus obliquidens* and *Zonotrichia albicollis*.

Variant	SIFT	Polyphen-2	PROVEAN	Mutation Taster	Pathogenicity level of ACMG			Variant Classification
					Strong	Moderate	Supporting	
<i>TUBB1</i> c.952C>T	0	1	-5.42	disease causing	PS4	PM2 PM6	PP3	Likely pathogenic

Table 2. Pathogenicity level of ACMG of TUBB1 c.952C>T

A

B

Figure 3A. The expression of p-EGFP, the TUBB1 R318W mutant and WT was analysed by qRT-PCR in human thyroid cells transfected with the control vector pEGFP-C-Flag, mutant TUBB1 c.C952T and WT for 24 hours. The data are expressed as means±SD from at least three separate experiments.

Figure 3B. Protein expression of TUBB1 in Nthy-ori 3.1 cells transfected with the control vector pEGFP-C-Flag, mutant TUBB1 c.C952T and WT for 72 hours.

A

B1

0 h 10 h 24 h

B2

Figure 4A. Effect of the TUBB1 mutation (p.R318W) on the proliferation of human thyroid cells. Nthy cells were transfected with 100 ng of the TUBB1-tagged EGFP vector, control vector pEGFP-C-Flag and mutant R318W-TUBB1 for 46 h, 72 h, 96 h, and 120 h, and MST-8 was used to detect the OD 490 value. * $P < 0.05$ compared with pEGFP-TUBB1. The data are expressed as means $\pm$ SD from at least three separate experiments.

Figure 4B. Effect on migration of Nthyori 3.1 cells transfected with the TUBB1-tagged EGFP vector, control vector pEGFP-C-Flag and mutant R318W-TUBB1 for 24 h, analysed by the wound healing assay for 0 h, 10 h, and 24 h.

B1. The cells were photographed at 0 h, 10 h, and 24 h using the cellSens Standard system. From top to bottom are pEGFP、pEGFP-TUBB1.C952T、and pEGFP-TUBB1. The length of the white line in the lower right corner of each picture is 200 μ m.

B2. No significant difference was found in the migration distance between pEGFP-TUBB1.C952T and pEGFP-TUBB1.