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Clinical and Genetic Analysis of a Compound Heterozygous Mutation in the Thyroglobulin Gene in a Chinese Twin Family With Congenital Goiter and Hypothyroidism

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Mutations in the thyroglobulin (TG) gene, which has an estimated incidence of approximately 1 in 100,000 newborns, cause autosomal recessive congenital hypothyroidism. The mutational spectrum of the TG gene and the phenotype–genotype correlations have not yet fully been established. We report a compound heterozygous mutation in the TG gene in a Chinese twin family with congenital goiter and hypothyroidism. We also describe the gene mutation associated with the genotype–phenotype of these children with congenital goiter and hypothyroidism. The whole coding sequence of the TG gene was analyzed by direct sequence, and the identified changes in the sequence were tested for benign polymorphism by denaturing high-performance liquid chromatography screening of the mutation and sequencing 200 chromosomes from normal controls. Analysis of the TG gene of the affected twin revealed a compound heterozygous mutation, including a novel missense mutation G2687A, which is predicted to result in a glutamine to arginine substitution at codon 877, and a known nonsense mutation C7006T, predicted to result in an arginine to stop codon at codon 2317. Analysis of 200 normal chromosomes did not identify the same change in healthy subjects. This is the first report of a TG gene mutation in the Chinese Han population. Our study provides further evidence that mutations in the TG gene cause congenital goiter and hypothyroidism, demonstrates genetic heterogeneity of the mutation, and increases our understanding of phenotype–genotype correlations in congenital hypothyroidism.

■ **Keywords:** TG gene, congenital goiter, hypothyroidism, compound heterozygous mutation, phenotype genotype correlations

Congenital hypothyroidism (CH) is one of the most common endocrine disorders in infancy. The disorder results in severe neurodevelopmental impairment and mental retardation if thyroid hormone therapy is not initiated within the first 2 months of life. It affects approximately one newborn infant in 3,000–4,000 live births (De Feliec & Di Lauro, 2004). In 80–85% of patients, the disorder is due to defects in early thyroid organogenesis, resulting in either absent or ectopic thyroid glands (dysgenesis). In a further 15% of patients, it is associated with inborn errors in thyroid hormone synthesis (dyshormonogenesis) (Macchia et al., 1998). A variety of research indicates that dyshormonogenesis in CH is often caused by mutations in one of the genes coding for the proteins responsible for thyroid hormone synthesis, such as the thyroglobulin (TG) (Medeiros-Neto, Targovnik, &

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Vassart, 1993), thyroperoxidase (TPO) (Abramowicz, Targovnik, et al., 1992; Abramowicz, Vassart, et al, 1992), sodium/iodide symporter (NIS) (Pohlenz et al.), pendrin (PDS) (Everett et al., 1997), $DUOX_2$ (Moreno et al., 2002), and $DEHAL_1$ genes (Moreno et al., 2008). Although these cases are often inherited in an autosomal recessive manner, large genetic heterogeneity was observed for phenotypes of this form of CH, ranging from euthyroid to severe goitrous hypothyroidism. In a child of non-consanguineous French–Canadian parents, CH with an in situ, normal-sized thyroid may have been due to a compound heterozygous loss-of-function mutation of the $DUOX_2$ gene (Hoste, Rigutto, Van Vliet, Miot, & De Deken, 2010), while a missense homozygous $DUOX_2$ mutation caused a large goiter in an adult patient (Ohye et al., 2008).

The TG gene maps on chromosome 8q24.2-8q24.3 (Bergé-Lefranc et al., 1985; Brocas et al., 1985) and contains an 8.5 kbp coding sequence which is divided into 48 exons, separated by introns varying in size up to 64 kbp (Mendive et al., 1999; 2001; Moya et al., 2000). The TG protein, produced predominantly by the thyroid and secreted from the endoplasmic reticulum into the follicular lumen through exocytosis (Dunn, Corsi, Myers, & Dunn, 1998), is the most abundant glycoprotein precursor to the thyroid hormones T_3 and T_4. Three transcription factors, TTF_1 (Javaux et al., 1992), TTF_2 (Zannini et al., 1997), and PAX8 (Zannini, Francis-Lang, Plachov, & Di Lauro, 1992), activate the promoter region of the TG gene to initiate transcription of TG and also to perform a variety of important physiological functions. TG acts as a substrate for thyroid hormone synthesis and also stores the inactive forms of thyroid hormone and iodine. Therefore, TG plays an important role in both the development and function of the thyroid gland. The abnormality of the TG gene is of great relevance to CH (Medeiros-Neto et al., 1993). Many countries have reported mutations in the human TG gene that are associated with congenital goiter with hypothyroidism (Targovnik, Esperante, & Rivolta, 2010). However, the mutational spectrum of the TG gene and the phenotype–genotype correlations have not been fully established, and no reports exist about TG gene mutations in the Chinese Han population.

In the present research, we focused on compound heterozygous mutations and describe the gene mutation associated with the genotype–phenotype of Chinese twins with congenital goiter and hypothyroidism caused by a mutation in the TG gene.

Methods

Patients

The subjects were twins from a Chinese family from the Shandong province who were identified through a neonatal screening program. Both twins were clinically diagnosed with congenital goiter and hypothyroidism, which was confirmed by biochemistry and ultrasound after an increased level of thyroid stimulating hormone (TSH) was detected in the screening program., Blood samples were collected from the twins after written informed consent was obtained.

DNA Analysis

Genomic DNA was extracted from peripheral blood leukocytes using standard methods. The human TG gene is encoded by 48 exons. Gene fragments covering the coding sequence, the flanking intronic sequence, the 5'UTR, and the 3'UTR of the TG gene (MIM#188450, GenBank NM_003235.4) were amplified using the primer pairs for exons 1–48 of the TG gene reported previously (Caron et al., 2003; Gutnisky et al., 2004). Identical amplification conditions were used in a total volume of 25 μl containing 250 nM dNTPs, 100 ng of template DNA, 0.5 μM of each primer, and 1.25 U AmpliTaq Gold DNA polymerase, in 1x reaction buffer (10 mM Tris HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$). PCR amplifications were performed with an initial denaturing step at 94 °C for 5 min, then 35 cycles as follows: 94 °C for 30 s, 51–65 °C for 60 s, 72 °C for 30 s, followed by 10 min of final extension at 72 °C. Amplified PCR products were purified and sequenced using the appropriate PCR primers and the BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA), and run on an automated sequencer, ABI 3730XL (Applied Biosystems), to perform mutational analysis.

DHPLC Screening of the Mutation in the TG Gene

Mutation screening in exon 10 and exon 40 of the TG gene was performed with denaturing high-performance liquid chromatography (DHPLC) (Wave DHPLC ; Transgenomic, Omaha, NE, USA) in 100 normal controls. The fragment harboring the c.2687 G>A and c.7006 C>T of the TG gene mutation was amplified. DHPLC was performed as follows: initial concentration at 48% of buffer A (0.1 M triethylammonium acetate (TEAA; Transgenomic), and 52% of buffer B (0.1 M TEAA containing 25% acetonitrile; Transgenomic) at 65 °C. Data were analyzed by comparison of the chromatograms.

Results

Clinical Phenotype

Detailed clinical information for all affected individuals is presented in Table 1. Each twin had an increased serum level of TSH when screened after birth, which was accompanied by a decreased serum T4 level. The only clinical sign of hypothyroidism was goiter. They were started on Euthyrox treatment at a 1 month of age, which was continued until they were 12 months old, when serum TSH and T4 returned to normal levels. At 15 months, their growth and development was normal, with no evidence of goiter.

TABLE 1

Clinical Analysis of Twins with Congenital Goiter and Hypothyroidism

	Twin 1	Twin 2
Age	1.5 years	1.5 years
Sex	Male	Male
Birth weight (kg)	2.55	2.30
Feeding	Breast feeding	Breast feeding
Preliminary diagnosis		
TSH (μIU/ml) [1.36–5.8]	> 100	> 100
T3 (μMol/L) [1.3–3.1]	1.06	1.15
T4 (nMol/L) [66–181]	21.22	21.50
FT3 (pMol/L) [2.8–7.1]	< 0.4	< 0.4
FT4 (pMol/L) [11.6–22.7]	2.46	2.55
Ultrasound (cm)		
Left lobe	0.9	0.7
Right lobe	0.3	0.3
Isthmic portion	0.3	0.3
After Euthyrox therapy		
1 month		
TSH (μIU/ml)	8.83	17.88
FT4 (pMol/L)	25.15	26.38
3 months		
TSH (μIU/ml)	10.6	14.14
FT4 (pMol/L)	24.76	24.83
6 months		
TSH (μIU/ml)	33.66	27.29
9 months		
TSH (μIU/ml)	7.47	10.07
12 months		
TSH (μIU/ml)	5.1	5.3
At 15 months	normal development	normal development

Note: Ranges in square brackets are normal ranges; TSH = thyroid stimulating hormone, T3 = thyroid hormone T3, T4 = thyroid hormone T4, FT3 = free T3, FT4 = free T4.

Mutation Analysis

Results of Mutation Analysis of TG gene. Analysis of the TG gene of the twins revealed a compound heterozygous mutation, including a novel missense mutation G2687A, which was predicted to result in a glutamine to arginine substitution at codon 877 in exon 10 (accession no.NM_003235.4; numbering the first nucleotide of the initiation codon as 1); and a known nonsense mutation C7006T, predicted to result in an arginine to stop codon at codon 2317 in exon 40 (Figure 1).

DHPLC Screening of the Mutation in the TG Gene. DHPLC analysis of 200 normal chromosomes from healthy control subjects of the same ethnic origin did not identify the same change (Figure 2). Therefore, this compound heterozygous mutation is possibly a causal mutation responsible for the phenotype of congenital goiter and hypothyroidism in this family.

Bioinformatic Analysis of the TG Gene Mutation. From the NCBI (National Center for Biotechnology Information) and UCSC (University of California Santa Cruz) websites, we obtained the TG family protein sequences. We used the Vector NTI software to obtain multiple-sequence alignments of TG family proteins in various species, including *Canis spp.*, *Danio rerio*, *Mus musculus*, *Rattus norvegicus*, *Sus scrofa*, rodent species, and humans (*Homo sapiens*) which is also the species list in Figure 3 legend. We found that codon 877 and codon 2317, where a compound heterozygous (p.R877Q and p. R2317X) mutation occurred, was located in a highly conserved region of the TG protein. From the online Protscale program, we compared hydrophobicity between wild-type and mutant TG (p.R877Q). We also used CLC Protein Workbench 3 (version 3.0.2) to predict the effect of the substitution on TG hydrophobicity. We found that the hydrophobicity of the mutant protein increased, which could be due to structural changes in the protein molecule that alter the normal protein folding, assembly, and biosynthesis of the thyroid hormones .

Discussion

If congenital goiter and hypothyroidism are often associated with TG gene defects that show classical Mendelian autosomal recessive inheritance, then the patients should be homozygous or compound heterozygous for TG gene mutations. Since the first description of TG gene mutations in patients with congenital goiter and hypothyroidism (Ieiri et al., 1991), at least 50 different mutations have been found scattered in most of the coding exons of the human TG gene: including 23 missense mutations,10 nonsense mutations, five single and one large nucleotide deletions, one single nucleotide insertion, and 10 splice site mutations (Machiavelli et al., 2010; Targovnik et al., 2010). These findings suggest that the recessive form of this disease results, to different degrees, from loss of function of normal protein. Changes in the functional structure of the TG protein affect the protein folding, assembly, and biosynthesis of the thyroid hormones, which reduces the cells' ability to export the protein from the endoplasmic reticulum.

In this work, we report a compound heterozygous mutation in the TG gene, with a novel substitution of glutamine to arginine at codon 877 (p.R877Q) in exon 10 and a known nonsense mutation in an arginine to stop codon at codon 2317(p.R2317X) in exon 40, found in twins of a Chinese family with congenital goiter and hypothyroidism. The primary structure of the human TG protein consists of eight domains: three families of cysteine-rich repetitive units (types 1, 2 and 3), one acetylcholinesterase (ACHE) homology region, and four hormonogenic acceptor tyrosines (Park & Arvan, 2004). Type 1 repetitive units present in the TG sequence have been suggested to act as binders and reversible inhibitors of the proteases implicated in TG processing (Molina, Pau, & Granier, 1996). The ACHE-homology domain is essential for protein dimerization, which plays an important role in the normal conformational maturation and intracellular transport of TG (Lee, Wang, Di Jeso, & Arvan, 2009). Cysteine residues are required in the tertiary structure of TG (Lee, Wang, Di Jeso, & Arvan, 1993).

This novel missense mutation TG c.G2687A (p.R877Q) identified in this study, just located the repetitive units of

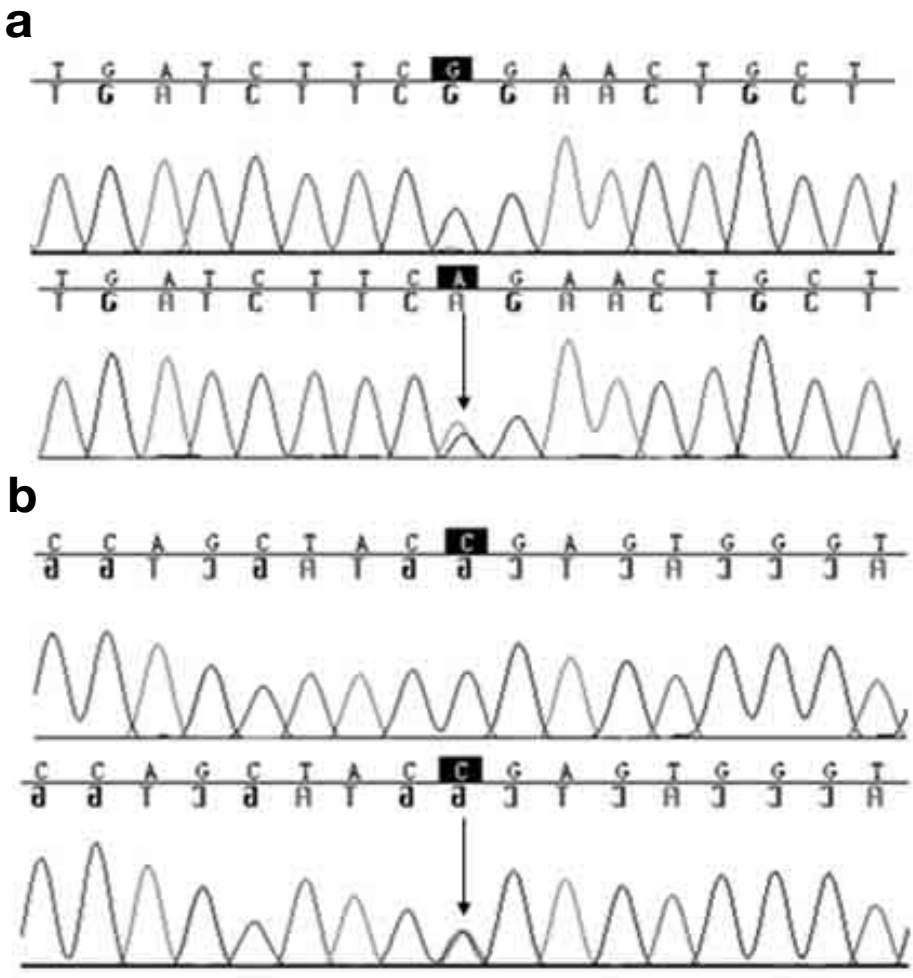

FIGURE 1

Partial sequence of exon 10 and exon 40 in the TG gene from normal and affected individuals. (a) Arrowhead indicates the heterozygous G and A at nucleotide 2687 in an affected patient; (b) Arrowhead indicates the heterozygous C and T at nucleotide 7006 in an affected patient.

Type 1 TG domains. Multiple-sequence alignments of TG family proteins in various species indicated that p.R877Q was located in a highly conserved region of the protein. This mutation could cause a reduction in inhibitors of cysteine proteases and binding partners of heparin, which might lead to a functional defect in the TG protein.

A nonsense mutation, p.R2317X, the cysteine to thymine heterozygous transition at nucleotide position 7006 (c.C7006T), creates a premature termination codon at the 2317 amino acid position, with the formation of a truncated TG protein. The functional consequence of this mutation is located in the ACHE-homology domain, which could cause defective conformational maturation and intracellular transport of TG. Because this mutation has also been found in an Argentinian family with congenital goiter and hypothyroidism, that is, an identical mutation in a different ethnic group of people, this indicates that p.R2317X is a 'hot spot' of mutation (Lee et al., 1993).

With the increasing number of reported cases of TG gene mutation, genotype–phenotype correlations have become

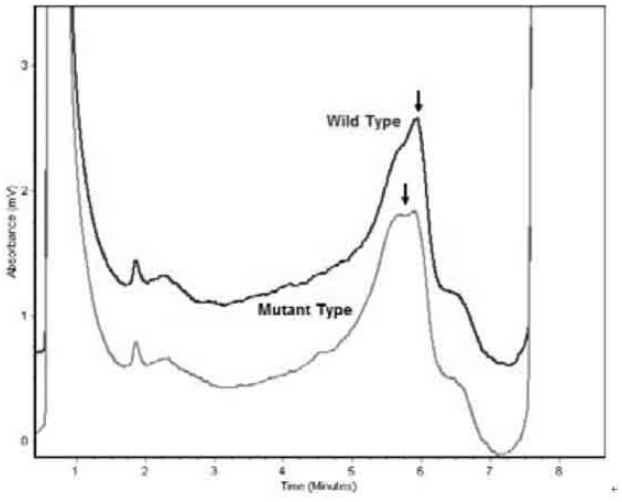

FIGURE 2

Denaturing high-performance liquid chromatography (DHPLC) shows wave pattern of wild type and mutant type TG gene. Note: wild type from normal individuals, mutant type from affected individuals.

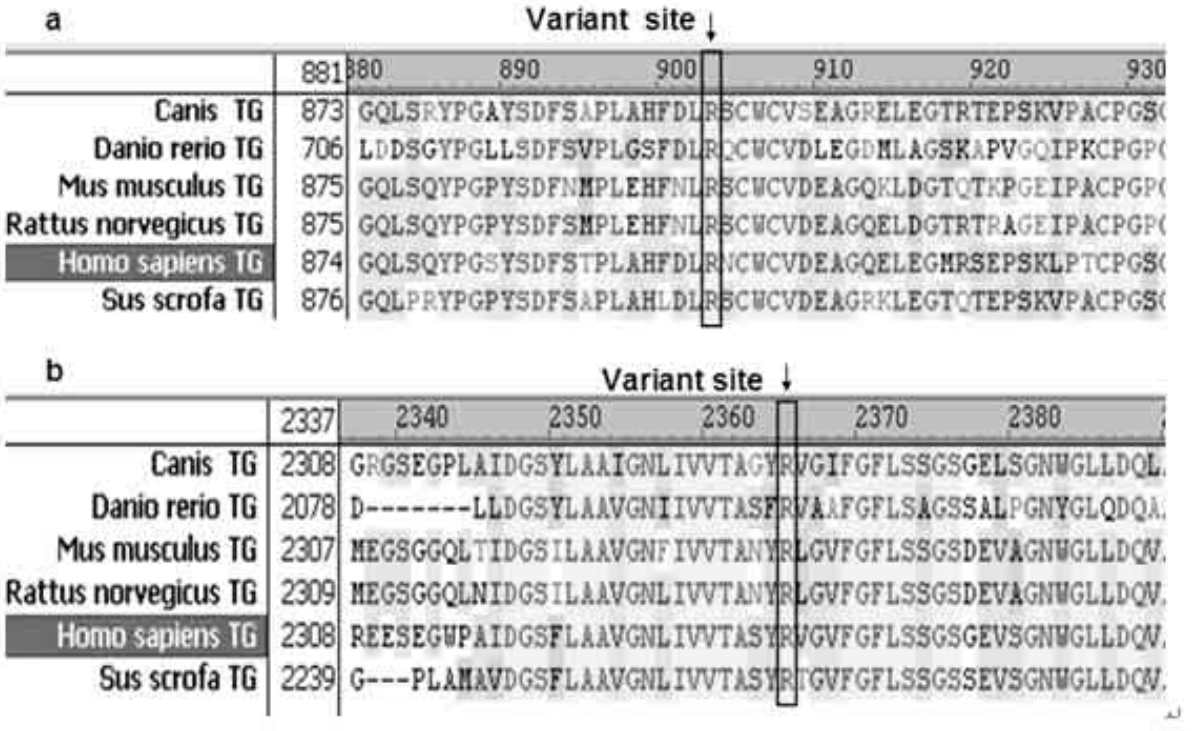

FIGURE 3

Multiple-sequence alignment of the TG protein from *Canis spp., Danio rerio, Mus musculus, Rattus norvegicus, Sus scrofa*, rodent species, and humans (*Homo sapiens*). Note: (a) The Arg 877 residue is located within a highly conserved region; (b) The Arg 2317 residue is located within a highly conserved region.

more complex than initially anticipated. The clinical spectrum of patients with TG mutation ranges from moderate to severe goitrous hypothyroidism. Even phenotypic variations among patients with the same mutations have been observed. The majority of patients have congenital goiter or goiter appearing shortly after birth. The phenotype of the affected twins observed in this Chinese family was relatively mild; they had no clinical signs of hypothyroidism other than goiter. They started Euthyrox treatment at 1 month of age, which was continued until they were 12 months old, when their serum TSH and T4 returned to normal levels. At 15 months, their growth and development were normal, with no evidence of goiter. However, the absence of clinical signs is presumably due to the fact that the patients were detected and treated in the neonatal period as the result of abnormal neonatal screening. Lee et al. (1993) reported a case with a compound heterozygous mutation of the TG gene for c.886C>T/c.4588C>T (p.R277X/p.R1511), while Caputo et al. (2007) reported a c.886C>T/g.IVS34-1G>C mutation of the TG gene in a non-consanguineous Brazilian family (Caputo et al., 2007). The phenotypes of two affected siblings and a nephew were relatively severe: cretinous faces, a multinodular goiter, slow reflexes, short stature, and coordination and speech difficulties. In a non-consanguineous Argentinean family with the same mutation (p.R2317X), a female patient with a birth weight of 3.03 kg had no clinical signs of hypothyroidism except a small goiter and high TSH levels on neonatal screening. However, after thyroxine treatment for 3 years, her hypothyroidism with goiter and low levels of TG remained. After treatment was reinitiated, her growth and development were normal with no evidence of goiter (Caputo et al., 2007). Compared with the clinical characteristics of the family we studied, the phenotype of the Argentinean case was relatively severe.

In conclusion, we reported a twin case of large goiter and hypothyroidism in a Chinese family, which was caused by a compound heterozygous mutation of the TG gene, including a novel missense mutation (p.R877Q) and a recurrent nonsense mutation (p.R2317X). The phenotype of these twins was moderate in comparison with that of other reported cases. To our knowledge, this is the first report of TG gene mutation in the Chinese Han population. Our study supports other evidence that mutations in the TG gene cause CH. Our findings also demonstrate genetic heterogeneity of the mutation and add to knowledge of the correlations of phenotype–genotype in CH. Such knowledge might assist in understanding the mechanism of the pathophysiology of CH, and form the basis of more accurate and rapid diagnosis (in the early stages of pregnancy) of this hereditary birth defect.

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