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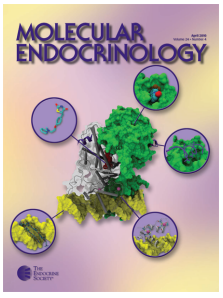

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New Insights into Thyroglobulin Pathophysiology Revealed by the Study of a Family with Congenital Goiter

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Context: Thyroglobulin (*TG*) gene mutations cause congenital hypothyroidism (CH) with goiter. A founder effect has been proposed for some frequent mutations. Mutated proteins have a defect in intracellular transport causing intracellular retention with ultrastructural changes that resemble an endoplasmic reticulum storage disease.

Objective: To reveal new aspects of thyroglobulin pathophysiology through clinical, cellular, molecular, and genetic studies in a family presenting with CH due to *TG* mutations from Galicia, an iodine-deficient area of Spain.

Design: The included clinical evaluation of family members, DNA sequencing for *TG* gene mutation and haplotyping analysis, ultrastructural analysis of thyroid tissue specimens from affected subjects, analysis of effects of mutations found on *TG* gene transcription, and *in vitro* studies of cellular production and secretion of mutated proteins.

Setting: Locations included primary care and university hospitals.

Results: Family members with CH, mental retardation, and goiter were compound heterozygous for c.886C→T (p.R277X) and g.IVS35+1delG. For c.886C→T, a founder effect cannot be excluded, and its transcription was hardly detectable. g.IVS35+1delG caused an in-frame deletion in exon 35 and produced a protein that, although synthesized, could not be secreted. Ultrastructural analyses showed morphological changes consistent with an endoplasmic reticulum storage disease.

Conclusion: The shorter thyroglobulin resulting from the novel g.IVS35+1delG was retained within the endoplasmic reticulum of thyrocytes, and together with p.R227X caused severe hypothyroidism with goiter. p.R277X, the most commonly described *TG* mutation, is caused by a *TG* exon-7 highly mutation-prone region, and the possibility that some cases were introduced to South America from Galicia cannot be excluded. **(*J Clin Endocrinol Metab* 95: 3522–3526, 2010)**

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Abbreviations: −Ex35, Exon 35 deletion; RER, rough endoplasmic reticulum; Tg, thyroglobulin.

Mutations in the human thyroglobulin (*TG*) gene cause dyshormonogenesis resulting in phenotypes ranging from euthyroid to severe goitrous hypothyroidism (1). *TG* codes for thyroglobulin (Tg), a large monomeric protein that serves as a precursor for thyroid hormone synthesis (2). Eighty percent of Tg has three repeated regions comprising cysteine-rich repeat domains covalently bound by disulfide bonds (2); the remaining constitutes a carboxyl-terminal domain homologous to acetylcholinesterase (3, 4) that functions as an intramolecular chaperone and escort for the three repeated regions (5). Tg conformational maturation culminates in Tg homodimerization with progression to a compact ovoid structure (6). Several chaperones in the rough endoplasmic reticulum (RER) interact with Tg during its maturation, preventing the export of improperly folded Tg proteins by a process known as RER-associated degradation (7–9). Correctly folded Tg homodimers are glycosylated in the Golgi and secreted as a dimer into the follicular lumen where specific tyrosyl residues are iodinated and coupled to form T_4 and T_3 (9).

To date, 43 inactivating mutations have been reported in the human *TG* gene (10), some of which cause exon deletions resulting in defective Tg proteins with only residual functional activity (4, 9). Some Tg mutants have defective intracellular transport (7–11) and accumulate within the RER, leading to congenital goitrous hypothyroidism with Tg deficiency being considered an RER storage disease (7, 8).

Here we report a family from Galicia, an iodine-deficient area in northwest Spain, with several members affected by congenital hypothyroidism and goiter caused by *TG* gene mutations. One mutation, c.886C→T (p.R277X), has been previously reported in families from Brazil and Argentina (12); these countries have a large immigrant Galician population, raising the question of whether an ancestor from Galicia may have introduced the p.R277X mutation to South America. A new mutation, g.IVS35 + 1delG, affecting the donor splice site of exon 35 is also reported; this mutation results in mRNA transcripts lacking exon 35, which therefore results in an unsecreted mutant Tg protein that accumulates in the cells.

Subjects and Methods

Subjects

The propositus, a 31-yr-old male, was referred to our clinic with hypothyroidism. At the age of 2 yr, he had been diagnosed with hypothyroidism, presenting with goiter, psychomotor delay, and growth retardation (Fig. 1A, subject II-4). The patient then developed a large goiter and mild mental retardation due to poor treatment compliance. The family pedigree is shown in Fig. 1; three of the eight propositus siblings have congenital hypothyroidism, goiter, and mild mental retardation (Fig. 1A, subjects II-7, II-8, and II-9), and one of the affected, a 30-yr-old female, previously had a total thyroidectomy due to a large multinodular goiter (Fig. 1A, subject II-9). Another sibling, a 40-yr-old euthyroid female, had a right hemithyroidectomy due to a large thyroid follicular adenoma (Fig. 1A, subject II-1).

Genetic studies

Sequencing of the *TG* gene and haplotyping were performed using lymphocyte DNA. For haplotyping, 19 markers were analyzed and compared with those from five Argentinian and one Brazilian family bearing c.886C→T [p.R277X] (12) (Supplemental Table 1 published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). The effect of the g.IVS35 + 1delG mutation on *TG* gene splicing and of c.886C→T and g.IVS35 + 1delG on *TG* gene transcription was investigated using cDNA from thyroid specimens of the two propositus sisters. The Institutional Review Board of the University of Santiago de Compostela School of Medicine approved the study, and informed consent was obtained from each individual. For further details, see Supplemental Methods.

Ultrastructural analyses

Electron microscopy was performed on thyroid surgical specimens obtained from the two sisters (Fig. 1A, subjects II-1 and II-9), one follicular adenoma and a normal thyroid tissue. Samples were fixed and postfixed in 2.5% glutaraldehyde and osmium tetroxide in sodium cacodylate buffer, respectively, and embedded in Spurr's epoxy resin, and ultrathin sections were stained with uranyl acetate-lead citrate.

Immunoblotting

Immunoblotting was performed on total extracts from frozen thyroid tissue as described previously (13) and in Supplemental Methods.

Site-directed mutagenesis of mouse Tg cDNA to create an exon-35 deletion mimic

Mutations were introduced into a mouse Tg cDNA (11) to create an exon-35 deletion mimic (details are given in Supplemental Methods). The transfection of this mutant cDNA results in expression of a protein lacking the *italicized* residues (P-*AVWSDTPSFCPSAALQSLTEEK*-VT) of mouse Tg that are homologous to the residues missing in exon 35-deleted human Tg (P-*IAQNNAPSFCPLVVLPSLTEKV*-SL).

Cell culture and transfection, metabolic labeling, and immunoprecipitation

HEK293 cells were cultured and transiently transfected with Tg plasmid cDNA as previously described (11). Transfected cells were starved for 30 min in Met/Cys-free DMEM and then pulse labeled with 180 μCi/ml ^{35}S-labeled amino acids (MP Biomedicals, Irvine, CA). The labeled cells were then washed with an excess of cold Met/Cys and chased in complete DMEM. After 5 h, the media were collected and the cells were lysed (11). Tg was immunoprecipitated with anti-Tg antibodies overnight at 4 C, and the radiolabeled immunocomplexed Tg was then recovered by precipitation with protein A-agarose (Sigma Chemical Co., St. Louis, MO). Immunoprecipitates were washed three times, boiled in sodium dodecyl sulfate sample buffer, resolved by SDS-PAGE, and analyzed by fluorography.

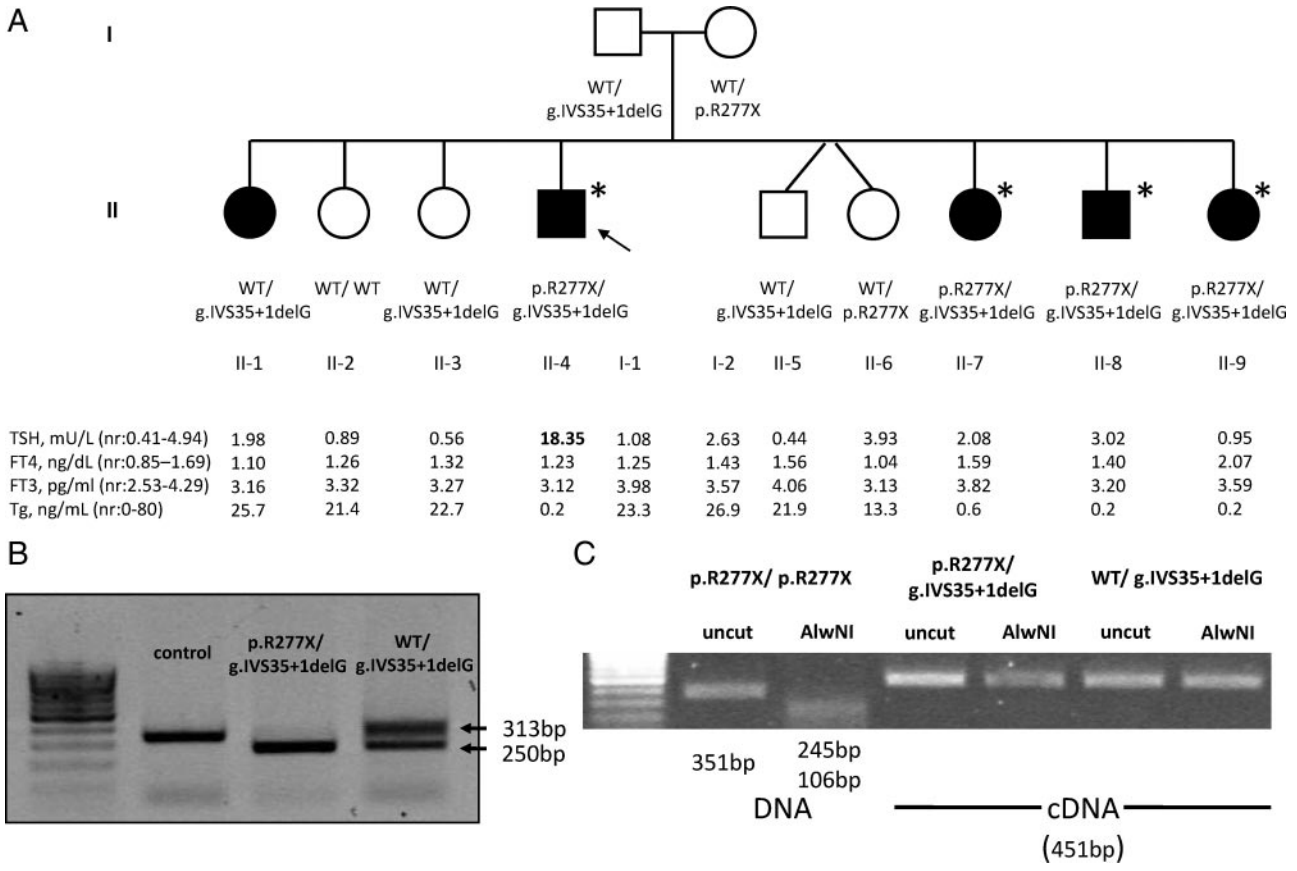

FIG. 1. A, Family pedigree, genotype, and serum levels of TSH, free T_4 (FT4), free T_3 (FT3), and Tg of all family members. Subjects II-4, II-7, II-8, and II-9 were receiving thyroid hormone replacement therapy. The propositus father was heterozygous for g.IVS35 + 1delG and the mother heterozygous for c.886C→T. The other family members with congenital goitrous hypothyroidism were compound heterozygous for c.886C→T and g.IVS35 + 1delG (subjects II-7, II-8, and II-9). Three members of the family were heterozygous for g.IVS35 + 1delG, including the member with a thyroid follicular adenoma (subjects II-1, II-3, and II-5); one member was heterozygous for c.886C→T (subject II-6); and one member was wild type (WT; subject II-2). *Arrow* indicates the propositus. *Black boxes* indicate goiter. *Asterisks* indicates family members with congenital hypothyroidism, goiter, and mild mental retardation. B, PCR amplification of thyroid tissue specimens. cDNA extending from exon 34 to exon 36 showed the expected 313-bp fragment in the wild-type subject and two fragments in WT/g.IVS35 + 1delG heterozygous subjects, a 313-bp fragment corresponding to the wild-type allele, and a 250-bp fragment corresponding to the allele causing exon 35 skipping; however, in the c.886C→T/g.IVS35 + 1delG compound heterozygote, only a 250-bp fragment was obtained. C, Restriction analysis with *Alw*NI, an enzyme that excises the fragment containing the c.886C→T mutation, on cDNA fragments extending from exon 6 to exon 8, showed no excision in the control or WT/g.IVS35 + 1delG heterozygote; in the c.886C→T and g.IVS35 + 1delG compound heterozygous subject a very small decrease in fragment intensity was observed after restriction, and no second fragment was observed. The efficiency of *Alw*NI was high, as shown by a 100% excision of a DNA fragment amplified from a patient homozygous for c.886C→T. The results shown in B and C indicate that the amount of mRNA produced by the allele bearing the c.886C→T mutation was lower than that of the wild-type allele.

Results

Genetic studies

The propositus was a compound heterozygous for c.886C→T and g.IVS35 + 1delG (Fig. 1A, subject II-4). c.886C→T causes a change from arginine (CGA) to a stop codon (TGA) at residue 277, p.R277X. The novel g.IVS35 + 1delG constitutes a deletion of guanine at the donor splice site of intron 35. Family genotype, serum thyroid hormones, and Tg levels are shown in Fig. 1A.

The haplotype of the Galician and South American families is shown in Supplemental Table 1. The Galician family differs from the Brazilian family with respect to six markers. Compared with the Argentinian families, there were differences in the Galician family with respect to four markers in family BA, two markers in family RM (allele 1), and one marker in families LD, RS, and RM (allele 2); there were five possible differences between family ME and the Galician family.

The g.IVS35 + 1delG mutation caused an in-frame loss of exon 35 in *TG* gene and generated a polypeptide that had lost 21 amino acids compared with the wild-type Tg.

TG mRNA expression was affected by genotype (Fig. 1, B and C). In the heterozygous for c.886C→T/g.IVS35 + 1delG, after amplification of cDNA fragments from exon 34 to exon 36, only a 250-bp product, corresponding to transcripts without exon 35, was observed instead of the expected 313-bp wild-type product from the c.886C→T allele (Fig. 1B). The patient expressed eight times fewer mRNA *TG* transcripts containing exon 35 than did either the normal control or WT/g.IVS35 + 1delG heterozygous. PCR ampli-

fication of the same cDNAs between exon 6 and exon 8 followed by restriction analysis with *Alw*NI showed very little decrease in band intensity in fragments obtained from c.886C→T/g.IVS35 + 1delG heterozygous (see Fig. 1C), indicating that only a small amount of the allele bearing the c.886C→T mutation was present in those fragments.

Ultrastructural analyses

Most of the follicular cells from the c.886C→T/g.IVS35 + 1delG heterozygous (Fig. 1A, subject II-9) showed a dilatation in the RER that contained fine protein-like material (Fig. 2A, *left*). The dilated RER produced an apical band of cytoplasm, containing phagosomes characteristic of thyrocytes (Fig. 2A, *left*). In the WT/g.IVS35 + 1delG heterozygous (Fig. 1, subject II-1), the RER was dilated to a lesser degree (Figs. 2A, *right*). In both subjects, an enlarged amorphous basal lamina was found at the interface of the thyrocytes and underlying stroma (Fig. 2A).

The fate of exon 35-deleted Tg

HEK293 cells were transfected with plasmids encoding either wild-type mouse Tg or mouse Tg that lacks the amino acids corresponding to the exon 35 deletion (−Ex35).

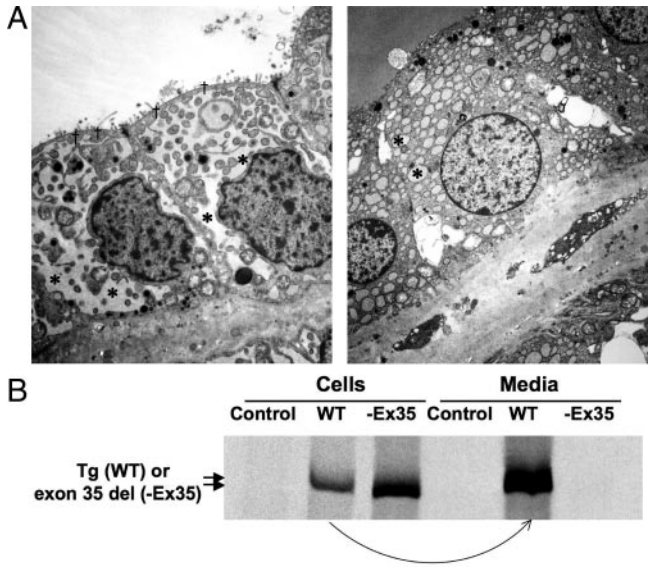

FIG. 2. A, Electron micrographs of the compound c.886C→T/g.IVS35 + 1delG (*left*) and wild-type (WT)/g.IVS35 + 1delG (*right*) heterozygotes. In the compound heterozygous tissue, a marked dilatation of RER (*), which produced an apical band of cytoplasm (†), was observed in follicular cells. The apical cell surface was covered by fine microvilli. In the WT/g.IVS35 + 1delG heterozygote the RER was also dilated, but to a lesser degree. An ample amorphous basal lamina was found in both cases. B, Fates of wild-type (WT) and exon 35-deleted (−Ex35) Tg. HEK293 cells were transiently transfected to express the constructs indicated. At 48 h after transfection, cells were pulse labeled with ^{35}S-labeled amino acids and chased for 5 h. The cells were lysed, and the chase media were collected. All samples were immunoprecipitated with anti-Tg and analyzed by reducing SDS-PAGE. Wild-type Tg was expressed and secreted efficiently (*arrow*), whereas −Ex35 was retained inside the cells and undetectable in the chase medium.

Within 5 h after synthesis, more than 70% of the wild-type Tg was secreted into the medium (*arrow*, Fig. 2B). Tg−Ex35 was also synthesized by HEK293 cells, but its secretion into the medium was undetectable (Fig. 2B, last lane).

Detailed immunoblotting results are given in Supplemental Results and Supplemental Fig. 1.

Discussion

We report a Galician family that has several members with congenital goitrous hypothyroidism caused by two different *TG* gene mutations: c.886C→T, a cytosine to thymine transition resulting in the change of an arginine to a stop codon at position 277 (p.R277X), and a novel mutation, g.IVS35 + 1delG, that causes the skipping of exon 35.

c.886C→T (p.R277X) is the most frequently reported *TG* gene mutation and has been previously found in families from Brazil and Argentina (1, 12, 14, 15), two countries with a large population of Galician immigrants. Although significant differences in haplotypes between families were observed, the possibility that the c.886C→T mutation has been introduced in South America by members of the Galician family cannot be excluded, because three families from Argentina showed a difference in only one of the 19 markers possessed by Galician family. c.886C→T occurs in a CpG-rich region that is prone to CT transversions due to deamination of 5-methylcytosine and its consequent replacement by thymine (16), an explanation for the relatively high frequency of this mutation.

c.886C→T does not create an alternative splicing site; the mutated *TG* mRNA transcript generates a truncated protein (14) that contains the Tg acceptor tyrosine 5 and the donor tyrosine 130 residues and can be glycosylated. Although functional studies have not been done on p.277X, studies using a smaller protein, p.C175X, have shown that it can be secreted. Those findings suggest that some form of a thyroid hormone can be produced by p.277X. Previous studies in homozygotes for c.886C→T have shown the presence of full *TG* mRNA transcripts in their thyroid tissues (17), suggesting that the absence of transcripts with the c.886C→T mutation in our patient is due to transcript degradation.

g.IVS35 + 1delG causes an in-frame deletion of exon 35 in *TG* mRNA transcripts, generating a polypeptide that has lost 21 amino acids from the wild-type Tg sequence with the introduction of a methionine at position 2067. The resulting protein is synthesized but cannot be released into the follicle lumen. Instead, this protein is retained and intracellularly processed. HEK293 cells transfected with a mouse Tg construct mimicking the human mutant were able to produce the mutated Tg−Ex35 protein, but no protein was detected in the culture medium. Correspondingly, an ultrastructural study showed an enlarged RER. Taken together, these find-

ings indicate that 1) Tg was retained in the RER of thyrocytes as previously reported for naturally occurring (7, 8, 11, 18) and *in vitro*-generated (19) Tg mutations, and 2) amino acids encoded by exon 35 are necessary for Tg secretion into the follicular lumen. Because human Tg has an even number of the cysteines, the loss of cysteine in position 2076 may contribute to Tg misfolding, preventing its secretion into the colloid.

In conclusion, we have described a family with several members having a goitrous hypothyroidism caused by c.886C→T and the novel g.IVS35 + 1delG *TG* gene mutations. Haplotype analysis cannot rule out the hypothesis that some cases with c.886C→T, the most commonly described *TG* mutation, were introduced to Argentina by members of this family. Although some defective Tg with the capacity to synthesize thyroid hormones might be produced from the allele with the c.886C→T mutation, we have shown that the faulty allele is not fully transcribed. Both mutated alleles, c.886C→T and g.IVS35 + 1delG, generate abnormal Tg proteins, and the resulting protein from the g.IVS35 + 1delG mutated allele cannot be not secreted but instead accumulates in the RER.

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Disclosure Summary: The authors have nothing to disclose.

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