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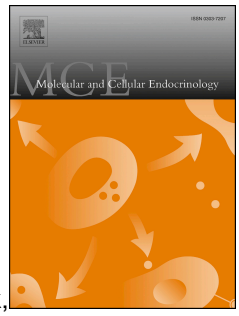

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Molecular Analysis of Thyroglobulin Mutations Found in Patients with Goiter and Hypothyroidism.

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Short title: Mutations in the Thyroglobulin Gene.

Key words: thyroglobulin gene, mutation, truncated thyroglobulin protein, goiter, hypothyroidism.

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Abstract

Thyroid dyshormonogenesis due to thyroglobulin (TG) gene mutations have an estimated incidence of approximately 1 in 100,000 newborns. The clinical spectrum ranges from euthyroid to mild or severe hypothyroidism. Up to now, one hundred seventeen deleterious mutations in the TG gene have been identified and characterized.

The purpose of the present study was to identify and characterize new mutations in the TG gene. We report eight patients from seven unrelated families with goiter, hypothyroidism and low levels of serum TG. All patients underwent clinical, biochemical and image evaluation. Sequencing of DNA, genotyping, as well as bioinformatics analysis were performed.

Molecular analyses revealed three novel inactivating TG mutations: c.5560G>T [p.E1835*], c.7084G>C [p.A2343P] and c.7093T>C [p.W2346R], and four previously reported mutations: c.378C>A [p.Y107*], c.886C>T [p.R277*], c.1351C>T [p.R432*] and c.7007G>A [p.R2317Q]. Two patients carried homozygous mutations (p.R277*/p.R277*, p.W2346R/p.W2346R), four were compound heterozygous mutations (p.Y107*/p.R277* (two unrelated patients), p.R432*/p.A2343P, p.Y107*/p.R2317Q) and two siblings from another family had a single p.E1835* mutated allele. Additionally, we include the analysis of 48 patients from 31 unrelated families with TG mutations identified in our present and previous studies. Our observation shows that mutations in both TG alleles were found in 27 families (9 as homozygote and 18 as heterozygote compound), whereas in the remaining four families only one mutated allele was detected. The majority of the detected mutations occur in exons 4, 7, 38 and 40. 28 different mutations were identified, 33 of the 96 TG alleles encoded the change p.R277*.

In conclusion, our results confirm the genetic heterogeneity of TG defects and the pathophysiological importance of the predicted TG misfolding and therefore thyroid hormone

formation as a consequence of truncated TG proteins and/or missense mutations located within its ACHE-like domain.

1.Introduction

Primary congenital hypothyroidism (CH) is the most common endocrine disease in children and one of the most common preventable causes of both cognitive and motor deficits (Park and Chatterjee, 2005; Rastogi and LaFranchi, 2010). The prevalence of CH is 1 in 2000-3000 live births. CH is an heterogenous group of thyroid disorders in which inadequate thyroid hormone production occurs due to disturbances in the gland organogenesis (thyroid dysembriogenesis, dysmorphogenesis or dysgenesis) or defects in proteins involved in any steps of thyroid hormone biosynthesis (thyroid dyshormonogenesis) (Park and Chatterjee, 2005; Rastogi and LaFranchi, 2010).

The dysembryogenesis results from a thyroid gland that is completely absent in orthotopic or ectopic location (agenesis or athyreosis), severely reduced in size but in the proper position in the neck (orthotopic hypoplasia) or located in an unusual position (thyroid ectopy) at the base of the tongue or along the thyroglossal tract (Abu-Khudir et al, 2017). Dysgenesis is associated with other major birth defects in 5–6% of cases. Genetic ascertainment is possible in a minority of cases and reveals mutations in genes responsible for the development or growth of thyroid cells: *NKX2.1* (also known as *TTF1* or *T/EBP*), *FOXE1* (also known as *TTF2* or *FKHL15*), *paired box transcription factor 8* (*PAX-8*), *NKX2.5*, and *TSHR* genes (Abu-Khudir et al, 2017; Grasberger and Refetoff, 2017)). Most of cases do not have an identifiable molecular defect in classical causative genes. Recently Kizys et al. (2017) reported DUOX2 gene mutations associated with CH by thyroid ectopy. This suggests that the DUOX2 N-terminal domain might be implicated in the etiology of thyroid dysgenesis. On the other hand, thyroid dyshormonogenesis has been linked to mutations in the *solute carrier family 5, member 5 transporter* (*SLC5A5*, encoding sodium iodide symporter, NIS) (Spitzweg and Morris, 2010; Targovnik et al, 2017), *solute carrier family 26, member 4 transporter* (*SLC26A4*, encoding pendrin, PDS) (Bizhanova and Kopp, 2010; Wémeau and Kopp, 2017), *thyroid peroxidase or thyroperoxidase, TPO)* (Ris-Stalper and Bikker, 2010; Targovnik et al,

2017), *DUOX2* (Grasberger, 2010, Muzza and Fugazzola, 2017), *DUOX2 maturation factor* (*DUOXA2*), *thyroglobulin* (*TG*) (Di Jeso and Arvan, 2016; Targovnik, 2012; 2017), *and iodotyrosine deiodinase* (*IYD*, also known as *iodotyrosine dehalogenase 1*, *DEHAL1*) (Moreno and Visser, 2010; Targovnik et al, 2017) genes. Except for Pendred's syndrome associated to *SLC26A4* mutations all forms of dysembryogenesis are nonsyndromic and not associated with other nonthyroidal anomalies.

TG is a large glycosylated protein secreted by the thyrocytes into the follicular lumen by exocytosis and it plays an essential role in the process of thyroid hormone synthesis. The human *TG* gene is a single copy gene of 270 kb long that maps on chromosome 8q24.2-8q24.3 (chr8: 133,879,203-134,147,147; GRCh37/hg19 assembly) and contains an 8,453 nucleotides in the coding sequence (GenBank Accesion Number: NM_003235.4) divided into 48 exons (Malthièry et al, 1987; Mendive et al, 2001; Mercken et al, 1985; van de Graaf, 2001). The human TG mRNA encodes a polypeptide chain of 2767 amino acids (Malthièry et al, 1987; Mercken et al, 1985; van de Graaf, 2001). A leader peptide of 19 amino acids is followed by a 2748 amino acid polypeptide, corresponding to the monomeric mature human TG. Eighty percent of the global TG monomer encloses repetitive motifs. The remaining 20%, that constitutes the carboxy-terminal part of the molecule, shows significant sequence conservation with the acetylcholinesterase (ACHE), therefore is named ACHE-like or ChEL domain (Malthièry et al, 1987; Mendive et al, 2001; Mercken et al, 1985; van de Graaf, 2001). Four hormonogenic acceptor tyrosine residues have been identified and localized at positions 5, 1291, 2554 and 2747 in human TG (Lamas et al, 1989; Dunn et al; 1998). The diagnostic criteria for TG defect is: intact iodide trapping, negative perchlorate discharge test and low serum TG levels. To date, one hundred seventeen mutations in the human *TG* gene have been identified and characterized associated to thyroid diseases: 19 splice site mutations, 23 nonsense mutations, 57 missense mutations, 13 deletions, 4 insertions or duplication and 1

imperfect DNA inversion (Abdul-Hassan et al., 2013; Agretti et al., 2013; Alzahrani et al., 2006; Baryshev et al., 2004; Brust et al., 2011; Cangul et al., 2014; Caputo et al., 2007a, 2007b; Caron et al., 2003; Citterio et al., 2011, 2013a, 2013b, 2015; Corral et al., 1993; Fu et al., 2016; Gonzalez-Sarmiento et al., 2001; Gutnisky et al., 2004; Hermanns et al., 2013; Hishinuma et al., 1999, 2005, 2006; Hu et al., 2016; Ieiri et al., 1991; Jiang et al., 2016; Kahara et al., 2012; Kanou et al., 2007; Kim et al., 2008; Kitanaka et al., 2006; Liu et al., 2012; Lof et al., 2016; Machiavelli et al., 2010; Medeiros-Neto et al., 1996; Mittal et al., 2016; Moya et al., 2011; Narumi et al., 2011; Nicholas et al., 2016; Niu et al., 2009; Pardo et al., 2008, 2009; Perez-Centeno et al., 1996; Peteiro-Gonzalez et al., 2010; Raef et al., 2010; Rivolta et al., 2005; Rubio et al., 2008; Targovnik et al., 1993, 1995, 2001, 2010b, 2012; van de Graaf et al., 1999). These mutations produce a heterogeneous spectrum of congenital hypothyroidism, with an autosomal recessive inheritance. Thereafter, the patients are typically homozygous or compound heterozygous for the gene mutations.

In the present study we report eight patients from seven unrelated families with CH, goiter, and low levels of serum TG. Molecular analyses identified three novel inactivating TG mutations: c.5560G>T [p.E1835*], c.7084G>C [p.A2343P] and c. 7093T>C [p.W2346R], and four previously reported mutations: c.378C>A [p.Y107*], c.886C>T [p.R277*], c.1351C>T [p.R432*] and c.7007G>A [p.R2317Q]. Aditionally, we describe the analysis of nature and frequency of TG mutations in 31 unrelated families with impaired TG function, characterized at molecular level in our laboratory, including the 7 families analyzed in the current work.

2. Materials and Methods

2.1. Patients

Patients with goiter, hypothyroidism, elevated serum TSH, low total serum levels of T_4 with simultaneous low or normal serum levels of T_3, low serum concentration of TG and negative anti-TG and anti-TPO antibodies were selected to participate in this study. Laboratory tests are shown in Table 1. All the patients come from iodide-sufficient areas. Families H (H:II-2) and I (I:II-3) were followed at "Servicio de Endocrinología" of "Hospital de Niños Santísima Trinidad", families J (J:II-1), K (K:II-1) and L (L:II-1) was followed at "División Endocrinología" of "Hospital de Niños Ricardo Gutiérrez", family LL (LL:II-1) was followed at "Servicio de Endocrinología" of "Hospital de Niños Sor María Ludovica" and family M (M:II-1 and M:II-2) was followed at "Clinique de Pédiatrie" of "Hôpital Jeanne de Flandre". The family pedigrees are shown in Figure 1.

Written informed consent was obtained from the parents of the children involved in this study, and the research project was approved by the institutional review board.

2.1.1. Family H

2.1.1.1. Patient H:II-2

The patient is the second child of an unrelated couple diagnosed at 27 days of life through the neonatal screening program being TSH: 59.1 mIU/L (cut off: 10). She was born in 2007 by caesarean section at term with appropriate weight for gestational age (3100 g). She had goiter, wide fontanelle, macroglossia and mild jaundice. Thyroid profile at the age of 35 days confirmed hypothyroidism with low TG (Table 1). The ultrasound showed enlarged thyroid gland globally, right lobe size: 22.5x15.4x10.6 mm, left lobe size: 28.7x14.6x13.5 mm and total volume: 4.84 ml (mean: 1.62±0.41, range: 0.7-3.3). Thyroid volume was calculated by multiplication of length, breadth and depth and a corrective factor (0.52) for each lobe (Perry et al., 2002). ^{99}Tc scintigraphy

showed an increased gland size with preserved distribution radiotracer uptake. She began L-T_4 replacement therapy (50 µg/day equivalent to 12 µg/kg/day) at 35 days of age normalizing thyroid function in the first subsequent control at 30 days of treatment started. TSH: 0,9 mIU/L (reference range: 0,85-7,79) and FT_4: 1,8 ng/ml (reference range: 1,01-2,09). She recieved the requiered treatment remaining euthyroid during regular testings. Growth and neurological development were normal. She attends primary school with good performance.

2.1.2. Family I

2.1.2.1. Patient I:II-3

The patient is the third girl of a non-consanguineous couple. Her mother has vitiligo, thyroiditis and hypothyroidism. The control during pregnancy was normal. She was born at term in 2010 after noncomplicated pregnancy, delivered by caesarean section, birth weight 3250 g. She was diagnosed by neonatal screening test at 11 days of life, TSH: 984 mIU/L (cut off: 10). The initial pediatric evaluation showed goiter, macroglossia, umbilical hernia and dry skin. At the age of 18 days, the diagnosis was confirmed (Table 1). Thyroid ultrasound showed hyperplasic gland in normal location, right lobe size: 22x12x14 mm, left lobe size: 21x11x14 mm and total volume: 3.6 ml (mean: 1.62±0.41, range: 0.7-3.3). The ^{99}Tc scintigraphy confirmed mildly enlarged thyroid gland with a normal uptake. L-T_4 therapy was started at 19 days with dose 50µg/day (12 µg/kg/day) with a very good compliance. Control in the 1st month of life showed normal thyroid function: TSH: 0.17 mIU/L (reference range: 0,85-7,79) FT4: 2,1 ng/ml (reference range: 1,01-2,09). Follow-up demonstrated normal growth and normal neurological development. She attends primary school with good performance.

2.1.3. Family J

2.1.3.1. Patient J:II-1

The patient, is the third child of an unrelated couple. Pregnancy was with gestational hypertension. Born at 37 weeks of gestation by cesarean section in 2013. Body weight: 2780 g. Neonatal screening positive for CH (TSH: 155 mIU/L, cut off: 10) and confirmation at 29 days of life (Table 1). Her palpable goiter with evidence of enlargement in the ^{99}Tc thyroid scan was the triggering factor to look for goitrous hypothyroidism due to defects in TG. She started treatment with L-T_4 at 29 days with 50 µg/day (12.7 µg/kg/day). Followed up till 2.9 years of age, thyroid gland is no longer palpable, grows in 75 percentile of height and 50 percentile of weight and develops normally.

2.1.4. Family K

2.1.4.1. Patient K:II-1

The patient, born in 2015, is the first child of non-consanguineous healthy parents without remarkable family history. Pregnancy occurred with gestational hypertension. Born at 37 weeks of gestation by cesarean section because of intrauterine growth restriction (IUGR) diagnosed by ultrasound. Body weight: 2655 g, height: 46 cm and APGAR score 4/10 at 1 & 5'. Positive for TSH newborn screening (TSH: >100 mIU/L, cut off: 10) was seen at 14 days of life (Table 1) when a goiter was evidenced in the ^{99}Tc thyroid scan. He immediately was supplemented with L-T_4 (37.5 µg/day; 13.8 µg /kg/day). At age of 2 years grows in 50th height percentile with normal weight, develops normally and remains under treatment clinically and biochemically euthyroid.

2.1.5. Family L

2.1.5.1. Patient L:II-1

This patient is the first child of a non-consanguineous healthy couple, born in 1994 after a 3rd normal controlled pregnancy by caesarean section. Her mother had 2 children from a previous couple, one who died at birth due to maternal hypertension and a healthy girl. Birth weight was 2580 g with unremarkable perinatal history. She presented delayed development with a later acquisition of developmental milestones. Walked at 24 months receiving support. At age 4.9 years she consulted to the endocrine centre for failure to thrive with short stature. She was at the 3rd percentile of weight and her height was -4.1 SD from normal argentine growth charts. She looked pale and with hypothyroid. She had a soft diffuse goiter. Her thyroid profile showed hypothyroidism (Table 1). Thyroid scan with ^{99}Tc evidenced a diffuse goiter and thyroid ultrasound showed enlarged thyroid gland (left lobe size: 8x10 mm, right lobe size: 31x11mm). Perchlorate discharge test was negative. With the suspicion of dyshormonogenetic goiter due to a probable defect in TG synthesis, she started at 4.9 years of age treatment with L-T_4 (50 µg/day; 3.4 µg /kg/day) with excellent response. Symptoms improved as well as biochemical markers achieving euthyroidism. She showed a catch up growth being in the 3rd percentile at age 7 and growing there afterwards according to her target height. However, she experimented a developmental delay. Her adherence to medication was rather good but with poor compliance to medical appointments. Puberty started at 10.7 years and completed normally. At the age of 14.6 years L-T_4 treatment was withdrawn for a month and she was reevaluated. Hypothyroidism was confirmed (Table 1). Ultrasonographic studies informed a thyroid gland with normal size, left lobe size: 28x9x13 mm, right lobe size: 28x7x11 mm and total volume: 2.8 ml (mean: 7.0±2.0). In her last visit at 14.8 years she was 150 cm height, normal weight and mildly retarded.

2.1.6. Family LL

2.1.6.1. Patient LL:II-1

The patient II-1, born in 1995, is the first child of an unrelated couple. Birth weight was 2100 g. Father born in Salta, Argentina, without thyroid diseases and mother born in Bolivia with menarche at 14 years and no familial thyroid diseases. He was referred to the endocrine centre at the age 12 years due to the presence of goiter (Figure 2). Four months before the consultation, he noted an augmented and sudden swelling without dysphagia. The only symptom referred was apathy. His family reports that goiter was progressive since 6 years of age. He had been treated with L-T_4 since the diagnosis of hypothyroidism, but after one year he stopped the treatment. Clinical examination reveals puffy facies, coarse voice, yellowish earthy pallor, dry skin and giant goiter with firm consistency in all the neck and supraclavicular hollow. Ultrasonographic studies evidenced a gland size augmented with multiple rounded echogenic images in both lobes (Figure 2), right lobe size: 114x51x52.4 mm, left lobe size: 109x48.3x49.6 mm and total volume: 294 ml (mean: 7.0±2.0). He is 153.4 cm tall and weights 43 kg. Cardiovascular control shows minimal pericardial effusion. Hypothyroidism was confirmed by laboratory tests (Table 1). Fine-needle aspiration biopsy showed hyperplasia in folicular epitelial cells flaps, with minimal focal anisocariosis. Treatment with L-T_4 was started (100 µg/day; 2.32 µg/Kg/day). He persisted with a big goiter, with irregular surface receiving L-T_4 at an adequate dose. Blood analysis indicated TSH: 4.47 mIU/L (reference range: 0.34-5.60), low TT_4: 1.8 ug/dl (reference range: 6.09-12.23), low FT_4: 0.22 ng/dl (reference range: 0.58-1.24) and normal TT_3: 284 ng/dl (reference range: 87-172). The serum TG concentration was low at 0.2 ng/ml (reference range: 1,6-80) suggesting that hypothyroidism could be related to defective TG synthesis. L-T_4 dose was increased to 125 µg/day. Total thyrodectomy was performed one year later due to the persistence of multinodular goiter. Macroscopic examination showed a piece weight of 229 g with irregular surface, predominantly solid light brown colour, with little cavities (Figure 2). The histopathological exam samples revealed folicles with epitelial hyperplasia, others with very much dilated lights of varied sizes between folicles septum of connective tissue

that clutter as nodules (Figure 2). In post-operative he showed hypocalcemia. Hypoparathyroidism was confirmed and he was treated with calcium carbonate and calcitriol. Despite of adjusts of the dose of L-T_4 the TSH concentrations persisted elevated and the levels TT_4 low. The patient's compliance to the treatment was poor.

2.1.7. Family M

They are the unique born from Northern French non-consaguineous parents. They both had a moderate goiter. The mother had TSH: 0.857 mU/L (reference range: 0.4-3.6), TG: 13.6 ng/ml (reference range: 1.5-43) and anti-TG antibodies: 0,75 mU/L (reference range: <1,5); the father had, TSH: 0.919 mIU/L, TG: 26.2 ng/ml and anti-TG antibodies: 0,84 mU/L.

2.1.7.1. Patient M:II-1

This index patient M:II-1 was born in 2003. A voluminous goiter with tracheal compression was detected by ultrasonography at the 24th week of gestation, with a very high level of TSH in cord plasma, 255 mIU/L (reference range: 0,23-3.8). Anti-thyroid antibodies (anti-TG, anti-TPO and TRAK) were negative. Five intraamiotic injections of L-T_4 were necessary to alleviate tracheal compression (dose: 500 µg per injection), respectively at the 25th, 27th, 29th, 33rd and 36th weeks of amenorrhea. A cesarean section was performed at the 37th week of gestation. She was immediately intubated before complete extraction and transferred to a neonatal resuscitation unit. Birth weight was 2870 g. In the neonatology unit, she presented a light stridor after removing the intratracheal tube. A goiter was noted clinically and by ultrasound, right lobe size: 32x16x14 mm, left lobe size: 31x14x9 mm and total volume: 5,7 ml (mean: 1.62±0.41, range: 0.7-3.3). Confirmation of hypothyroidism at 5 days of life (Table 1). ^{123}I scintigraphy confirmed the existence of a normally fixing thyroid gland, unhappily without any quantification. TG turned out to be undetectable, which led to the suspicion of a defect in its gene. L-T_4 (20 µg/day; 7.1 µg/kg/day) administration was

started since the day 5 and maintained continuously. Consequently, she developed a transient autistic syndrome. She remained well balanced, regarding clinical, biological and ultrasound thyroid data. A micronodule of a 2.6 mm was detected in the right lobe. At the last visit, at the chronological age of 13 years 8 months, she received 100 µg/day (2.4 µg/kg/day) L-T_4, was 163 cm tall (75^{th} percentile), weighed 42 kg and has menstruated since she was 12.9 years old. Her behavior and her school abilities are normal.

2.1.7.2. Patient M:II-2

Patient M:II-2, a sister of patient II-1, was born in 2005. A fetal goiter was detected at 19 weeks of gestation, cord TSH was 85 mIU/L (reference range: 0.23-3.28), cord TG undetectable. She received 8 intramniotic injections of 500 µg each of L-T_4, respectively at 23^{rd}, 25^{th}, 28^{th}, 30^{th}, 32^{nd}, 34^{th}, 35^{th}, 37^{th} weeks of amenorrhea. Contrarily to II-1, delivery was uneventful. Birth weight was 3000 g, birth length 48 cm, cranial circumference 35 cm. Confirmation of hypothyroidism at 3 days of life (Table 1), total thyroid volume at ultrasound: 4.1 ml (mean: 1.62±0.41, range: 0.7-3.3, right lobe size: 22x14x12 mm and left lobe size: 22x16x12 mm). TG plasma level was postnatally checked to be undetectable. She was initially treated by L-T_4 (25 µg/day; 8.3 µg/kg/day) since the 4^{th} day, treatment maintained continuously. She is well balanced, by an average dose of L-T_4 of 62.5 µg/day (2 µg/kg/day). She was 150 cm tall, weighed 31 kg and started puberty with a B2P1 stage at the chronological age of 11 years 5 months. Her behavior and capabilities are normal.

2.2. Laboratory testing

Serum TSH, serum total T_4 (TT_4), serum total T_3 (TT_3), serum free T_4 (FT_4), serum TG, anti-TPO antibodies and anti-TG antibodies levels were determined by electrochemiluminescence

immunoassay (ECLIA, Elecsys 2010, Roche Diagnostic Corporation, Indianapolis, IN, USA) and by quimioluminiscence immunoassay (ACCSSES Beckman Coulter, Brea, CA, USA).

2.3. Genomic PCR amplification.

Genomic DNA was isolated from peripheral blood leucocytes by using the cetyltrimetilammonium bromide (CTAB) method and stored at -20 C until analysis. The 180 bp of the promotor region and all 48 exons of the TG gene, including splicing signals and the flanking intronic regions were amplified using the primers and PCR conditions previously reported (Gutnisky et al., 2004).

2.4. DNA sequencing

TG PCR fragments were sequenced using sense and antisense specific primers or M13 universal primers reported previously (Gutnisky et al., 2004), with the Big Dyedeoxyterminator Cycle Sequencing Kit (Applied Biosystems, Weiterstadt, Germany). The samples were analyzed on the ABI Prism 3100 DNA sequencer (Applied Biosystems).

2.5. Bioinformatic analysis

Amino acid sequence homology between Homo Sapiens (National Center for Biotechnology Information (NCBI) reference sequence, accession number: NP_003226.4), Bos Taurus (NP_776308.1), Rattus norvegicus (NP_112250.2), Mus musculus (NP_033401.2), Xenopus tropicalis (NP_001316486.1; Holzer et al., 2016), Dano rerio (Zebrafish; NP_001316794; Holzer et al., 2016) and Petromyzon marinus (Lamprey; Holzer et al., 2016) TG species was compared using the CLUSTAL W (1.83) multiple sequence alignment (http://www.ch.embnet.org/software/ClustalW.html). The deleterious effect of missense mutations, identified in this study, were assessed using Polymorphism Phenotyping v2 (PolyPhen-2,

http://genetics.bwh.harvard.edu/pph2) and Sorting Intolerant From Tolerant (SIFT)/PROVEAN (http://provean.jcvi.org/genome_submit_2.php) prediction tools.

2.6. Nucleotide and amino acid nomenclatures

The genomic position corresponds to the GRCh37 assembly. The nucleotide position in human TG mRNA was designated according to NCBI reference sequences, accession number: NM_003235.4. The A of the ATG of the initiator methionine codon is denoted as nucleotide +1 (van de Graaf et al., 2001). The amino acid positions are numbered after subtracting the 19-amino-acid signal peptide (van de Graaf et al., 2001).

3. Results

3.1. Screening of mutations in the TG gene by direct sequencing analysis

To identify the deleterious TG mutations from the index patients of the seven unrelated families, all 48 exons of the TG gene, along with the flanking intronic sequences, as well as the TG promoter, were screened by direct DNA sequencing. A total of 15,000 bases were analyzed in each patient. Sequence analysis showed that the splicing consensus sequences (GT-AG) were rigorously conserved in all introns analyzed in the group of patients. Three novel inactivating TG mutations and four previously reported mutations were identified. One mutation, detected in index patients I:II-3 of family I, J:II-1 of family J and L:II-1 of family L, was a previously identified cytosine to adenine transversion at nucleotide position 378 (c.378C>A, Chr 8:133883696C>A) in exon 4, which replaces a tyrosine residue at position 107 by a premature stop codon [p.Y107*] (Figure 1) (Citterio et al., 2013). p.Y107* mutation is absent among the variants recorded in Genome Aggregation Database (gnomAD; http://gnomad.broadinstitute.org), NHLBI GO Exome Sequencing Project database (ESP; http://evs.gs.washington.edu/EVS) or dbSNP, database of single nucleotide polymorphisms and multiple small-scale variations (https://www.ncbi.nlm.nih.gov/snp) and was only previously reported as a compound heterozygous variant in an Argentinean patient (Citterio et al., 2013). The second mutation, found in index patient I:II-3 of family I, K:II-1 of family K and L:II-1 of family L was a extensively documented nonsense mutation involving a cytosine to thymine transition at nucleotide 886 in exon 7 (c.886C>T, Chr 8:133894854C>T, dbSNP: rs121912648, ClinVar ID: 12695), which replaces the wild-type arginine at codon 277 with a stop codon [p.R277*] (Figure 1). The p.R277* mutation has been described previously in heterozygosity or homozygosity in Argentinean (Caputo et al., 2007a; 2007b; Citterio et al., 2013; Machiavelli et al., 2010; Rivolta et al., 2005), Brazilian (Brust et al., 2011; Gutnisky et al., 2004; Pardo et al., 2009; van de Graaf et al., 1999) Galician (Peteiro-Gonzalez et al, 2010), British

(Nicholas et al., 2016), French (Citterio et al., 2011) and Iraquis (Abdul-Hassan et al., 2013) populations. This mutation was available in heterozygous state from gnomAD in Ashkenazi Jewish, Latino, Europeans (non Finnish), European (Finnish), African and South Asian populations with an estimated total Minor Allele Frequency (MAF) of 0.0003463 % for the allele ^{886}T (Table 2). Additionally, this allele was also identified in the ESP dataset in both European American (MAF: 0.0005813; mutated TG alleles/total TG alleles: 5/8,600) and African American (0.0006808; 3/4,406) ethnic groups, indicating the presence of this inactivating mutation in the general population.

The third mutation, identified in index patient H:II-2 of family H, was a known cytosine to thymine transition at nucleotide position 1351 in exon 9 (c.1351C>T, Chr 8:133898968C>T, dbSNP: rs773142559). Instead of encoding for an arginine residue at position 432, the triplet harboring the mutation encodes a stop codon [p.R432*] (Figure 1) (Niu et al., 2009; Kahara et al., 2012; Nicholas et al., 2016), present in 3 of 17,238 alleles analyzed in the East Asian population and 1 of 111,548 alleles in the European (non Finnish) population from gnomAD (Table 2). The fourth mutation, detected in index patient M:II-1 of family M was the novel p.E1835* mutation, located in exon 30, a single nucleotide substitution guanine to thymine at nucleotide 5560 (c.5560G>T, Chr 8:133978816G>T) that replaces a glutamic acid at codon 1835 with a stop codon (Figure 1). This variant is absent in gnomAD, ESP database and dbSNP. The fifth mutation, characterized in index patient J:II-1 of family J was an already known missense mutation in exon 40, where a guanine to adenine transition at nucleotide position 7007 (c.7007G>A, Chr 8:134034366G>A, dbSNP: rs121912650, ClinVar ID: 12705) produces the substitution of an arginine for glutamine at codon 2317 [p.R2317Q] (Figure 1) (Kitanaka et al., 2006 ; Hishinuma et al., 2006). This mutation, previously identified in a Japanese patient in association with a CH phenotype, it is present in 1 of 111,700 alleles analyzed for the minor allele ^{7007}A in the European (non Finnish) population and

absent in other ethnic groups including East Asians (Table 2) from gnomAD. p.R2317Q is absent in ESP database. The sixth mutation, characterized in index patient H:II-2 of family H was a novel missense mutation GCT>CCT in nucleotide 7084 (c.7084G>C, Chr 8:134042113G>C) leading to a substitution of alanine by proline [p.A2343P] in exon 41 (Figure 1). The latter mutation, identified in index patient LL:II-1 of family LL, also in exon 41, TGG>CGG at position 7093 (c.7093T>C, Chr 8:134042122T>C), substituting tryptophan by arginine in position 2346 [p.W2346R] (Figure 1). This mutation has not been described before. We ruled out the possibility that the p.A2343P and p.W2346R mutations could be polymorphisms because they were not detected as variations in the TG gene in gnomAD, ESP database and dbSNP.

3.2. Segregation analysis of the mutations in the TG gene

In family H, index patient H:II-2 was a compound heterozygous for c.1351C>T/c.7084G>C [p.R432*/p.A2343P] who inherited a copy of c.1351C>T mutation from his mother and a copy of c.7084G>C mutation from his father (Figure 1), whereas his unaffected brother H:II-1 was only carrying of the wild type alleles (Figure 1).

The index patient I:II-3 in family I was found to be a compound heterozygous for c.378C>A/c.886C>T [p.Y107*/p.R277*]. Direct sequencing of exon 4 and 7 of both parents' genomic DNA indicated that the patient inherited c.378C>A mutation from the mother and c.886C>T mutation from the father (Figure 1). Her sister I:II-1 and his brother I:II-2, were found to be heterozygous healthy carriers of c.378C> A (Figure 1).

In family J, index patient J:II-1 was a compound heterozygous for c.378C>A/c.7007G>A [p.Y107*/p.R2317Q]. Heterozygous mutations were detected in the father J:I-1 and mother J:I-2. The c.378C>A mutation was detected in the mother, while the c.7007G>A mutation was detected in the father (Figure 1).

In family K, index patient K:II-1 was homozygous for c.886C>T [p.R277*] (Figure 1). Analysis by sequencing of their parent showed that they are healthy heterozygous carriers of the mutation. To discriminate between a de novo recurrence of the p.R277* mutations and a founder effect in Argentinean patients, we compared the SNP haplotypes identified in the index patient K:II-1 from family K and those SNP haplotypes previously reported from the patients A:III-2 (family A, Citterio et al, 2013a) B:II-2 (family B, Citterio et al, 2013a), RM (Rivolta et al., 2005) and ME (Caputo et al., 2007a), carrying all the same p.R277* mutation also in homozygous state. The 15 exonic TG SNPs markers (c.229G>A, c.2200T>G, c.2334T>C, c.2488C>G, c.3082A>G, c.3474T>C, c.3935G>A, c.4506C>T, c.5512A>G, c.5995C>T, c.6695C>T, c.7408C>T, c.7501T>C, c.7589G>A and c.7920C>T) were used for haplotype analysis (Figure 3) (van de Graaf et al., 2001; Rivolta et al., 2005). The presence of exonic SNPs was evaluated by sequencing. The SNP analysis showed that the individuals affected K:II-1 (family K), A:III-2 (family A) and B:II-2 (family B) are homozygous for the same combinations of polymorphisms (Figure 3). This is a strong indication that the p.R277* alleles in three families might be derived from a common ancestral chromosome. However, comparative analysis between the SNP haplotype segregation from families K, A and B with RM and ME patients, showed one difference with patient RM (c.5995C>T [p.R1980W]) and three differences with the patient ME (c.5995C>T [p.R1980W], c.7501T>C [p.W2482R] and c.7920C>T [p.Y2621Y]) (Figure 3). These findings confirm that it is very likely that the p.R277* mutation is also an independent mutational event in Argentinean population.

The index patient L:II-1 in family L was found to be a compound heterozygous for c.378C>A/c.886C>T [p.Y107*/p.R277*] (Figure 1). Only the patient's mother was available for sequence analysis, and she was found to carry the c.886C>T mutation. The comparison of the 15 exonic TG SNPs markers identified in the index patient L:II-1 from family L and in the index

patient I:II-3 from family I, carrying the same compound heterozygous for c.378C>A/c.886C>T showed two clear differences (data not shown). L:II-1 from family L harbors T and T, in homozygous state, in the SNPs localized in the nucleotide positions 5995 (c.5995C>T [p.R1980W]) and 7501 (c.7501T>C [p.W2482R]). In contrast, I:II-3 from family I harbors C and C also in homozygous state in the same SNPs. This strongly suggested that the c.378C>A/c.886C>T genotype are due to independently recurrent mutations.

In family LL, the index patient LL:II-1 was homozygous for c.7093T>C [p.W2346R] (Figure 1). The maternal allele had the missense mutation. Father's DNA was not available.

Index patient M:II-1 in family M, as well as her affected sister M:II-2, are heterozygous for p.E1835* mutation. Both had clinical symptoms of CH and are currently being treated with T_4 supplementation. Because congenital hypothyroidism due to mutations in the TG gene are inherited in an autosomal recessive manner, the patients should be homozygous or compound heterozygous and the parents should be carriers of one TG mutation. Direct sequencing from M:II-1 suggesting the absence of one second mutation in the exonic coding or noncoding (5' and 3' UTR) sequences, the promoter region or the exon/intron boundaries of the TG gene. Unfortunately, the DNA of the mother and father was not available for the analysis. However, as the same allele is present in both siblings then it is not a de novo mutation and must have been present in one of the parents.

3.3. Bioinformatic data analysis

Four out of seven mutations identified in this study leads to a premature stop codon in TG polypeptide coding sequences, resulting in an early truncated protein with limited ability to generate thyroid hormones (Figure 4). The impaired function of this type of mutant proteins is well documented in the literature. On the contrary, the deleterious effect caused by the other three mutations identified in the ACHE-like domain needs greater precision (Figure 4). In this context,

the negative effect of the p.R2317Q, p.A2343P and p.W2346R mutations was evaluated in silico studies by assessing the degree of evolutionary conservation of the respective amino acids among several animal wild-type TGs and its pathogenic effects by prediction of the possible impact on the structure and function of the protein using straightforward physical and comparative considerations. Multiple sequence alignment of the human TG with sequences found in the NCBI (Bos taurus, Rattus norvegicus, Mus musculus, Xenopus tropicalis, Dano rerio and Petromyzon marinus), using Clustal method, revealed that wild-type arginine, alanine and tryptophan residues at positions 2317, 2343 and 2346, respectively, are strictly conserved in all TG species analyzed (Figure 5). It was suggested that these amino acids were important for the TG structure or its function.

p.R2317Q, p.A2343P and p.W2346R were predicted to be pathogenics by PolyPhen-2, PROVEAN and SIFT algorithms . Theses mutations are predicted to be probably damaging with a score of 1.00 for the three with PolyPhen-2, damaging with PROVEAN (cut off: -2.5; p.R2317Q: -3.91; p.A2343P: -4.63; p.W2346R:-13) and deleterious with SIFT (cut off: 0.05; p.R2317Q: 0.000; p.A2343P: 0.001; p.W2346R: 0.000).

3.4. Analysis of the nature and frequency of TG mutations in 31 unrelated families

The aim of this study was to analyze the nature and frequency of TG mutations in 48 patients associated with impaired TG function from 31 unrelated families genotyped in our laboratory. Therefore, 40 patients from 24 families previously published (Caputo et al., 2007a, 2007b; Caron et al., 2003; Citterio et al., 2011, 2013a, 2013b, 2015; Gutnisky et al., 2004; Machiavelli et al., 2010; Rivolta et al., 2005; Targovnik et al., 1993, 1995, 2001, 2010b, 2012) and 8 patients from 7 families analysed in the current study were evaluated. As shown in Table 3 six groups of mutations were observed: nonsense mutations (nine different ones: p.Y107*, p.R277*, p.R432*, p.Q717*, p.R768*, p.R1511*, p.Q1777*, p.E1835*, p.R2317*) which are the most frequently found (51 of the 96

studied TG alleles, i.e. 53.13 %); five missense mutations in the ACHE-like domain (p.A2215D, p.R2223H, p.R2317Q, p.A2343P, p.W2346R) found in 14 mutated TG alleles are second in frecuency (14.58 %); five mutations that putatively affect splicing (c.745+1G>A, c.4159+3_+4delAT, c.5686-1G>T, c.6200-1G>C, c.7036+2T>A) responsible for eight affected alleles are third in frecuency (8.33 %); while five frameshift mutations (p.L235Tfs*3, p.G362Gfs*21, p.R893Rfs*54, p.I1244Ifs*3, p.K1803Kfs*30) present in seven alleles are fourth in frequency (7.29 %); followed by one imperfect DNA invertion of 16,962 bp in the TG gene, associated with two deleted regions at both sides of the inversion limits, responsible for six affected alleles (6.25 %); and finally missense mutations involved in the wild type cysteine (three different ones: p.C164Y, p.C1262Y, p.C1981W) residues that affects three TG alleles (3.13 %). The mutations involved in these 31 families are further discussed in the *Discussion.*

4. Discussion

Based on Sanger sequencing analyses, we verified that three novel inactivating TG mutations: p.E1835*, p.A2343P, and p.W2346R, and four previously reported mutations: p.Y107*, p.R277*, p.R432* and p.R2317Q cosegregate with the congenital goitre and hypothyroidism phenotype. All affected individual have clinical and biochemical criteria compatible with CH associated with TG defects: low serum TG and high levels of serum TSH with simultaneous low serum total T_4 levels and low or normal serum T_3 levels. Very low TG serum concentrations is a key factor for the diagnosis of TG defects. Patients with thyroid dyshormonogenesis by TG gene mutations have a variable degree of primary hypothyroidism and thyroid gland enlargement depending on the severity of the defect and/or an adequate compliance to replacement therapy by the patient. Further, the amount of iodine intake influences thyroid function. In untreated patients, a complete defect causes a severe phenotype resulting in mental retardation with a large goitre. Unfortunately, this is the case of our patient LL:II-1 from family LL who needed psycho-pedagogical, phonoaudiological and/or psychomotor assistance. The ultrasonography was compatible with multinodular goiter and marked heterogeneity of the echographic responses with large hyperechoic nodules and the histopathological analyses showed follicles with epitelial hiperplasia and others with very much dilated lights (Figure 2). In contrast, the M:II-1 and M:II-2 siblings from family M with fetal goiter diagnosed by antenatal ultrasound and treated early with hormone replacement resulted in normal brain development and physical growth.

The TG protein is composed of four structural and functional regions (Figure 4) (Malthièry et al, 1987; Mendive et al, 2001; Mercken et al, 1985; van de Graaf, 2001). The N-terminal and the central part of the monomer include three types of repetitive motifs, called TG type-1, TG type-2, and TG type-3, organized in three regions (I, II and III), comprising Cys-rich repeats covalently bound by disulfide bonds (Figure 4). Interestingly, type-1 repeats could function as binder and

reversible inhibitors of the protease (Molina et al,1996). TG type-1 domains have been found as parts of six architecturally distinct protein groups (Novinec et al, 2006). Region I comprises 10 of the 11 TG type-1 repeats, a linker and hinge segments. Region II contains 3 TG type-2 repeats and the 11th TG type-1 repeat, whereas region III contains five TG type-3 repeats. The fourth region located between residues 2192 to 2716, is the ACHE-like domain (Figure 4) (Malthièry et al, 1987; Mendive et al, 2001; Mercken et al, 1985; van de Graaf, 2001). ACHE-like domain is required for protein dimerization and consequently plays a critical structural and functional role in the TG protein, that is essential for intracellular transport of TG to the site of its hormonogenesis (Park and Arvan, 2004; Lee et al., 2009, 2011; Lee and Arvan, 2011). This region functions as an intramolecular chaperone and as a molecular escort for TG regions I, II, and III (Lee et al., 2008). Newly synthesized *TG* is transported from the endoplasmic reticulum (ER) to the cell surface via the Golgi complex. All truncated forms detected in this study eliminate the ACHE-like domain in its entirety. p.Y107*, p.R277* and p.R432* mutants comprise only a part of region I (Figure 4), while p.E1835* includes regions I, II and only a part of region III (Figure 4). So far, 24 inactivating nonsense mutations that generate truncated proteins have been reported in the coding regions of *TG* associated to CH, particularly have been localized in exons 4, 7, 9, 10, 13, 20, 22, 27, 30, 31, 37, 40, 46 and 47 (Table 4). The primary functional consequences of nonsense mutations identified in the TG gene could be structural changes in the protein molecule that alter the biosynthesis of thyroid hormones. These truncated mutants have an impairment in thyroid hormone synthesis by loss of the carboxyl terminal hormonogenic sites at positions 2554 and 2747. However, all truncated proteins identified to date except p.Y107*, p.R140* mutants retain some ability for T_4 synthesis because these still harbor both the acceptor Tyr 5 (exon 2) and the donor Tyr 130 (exon 4) of the amino-terminal hormonogenic site (Lamas et al, 1989; Dunn et al; 1998). Recently, Citterio et al,. 2017 demonstrated than the carboxyl terminal end of intact TG contains “de novo” formed T_3,

independent of deiodinase activity. In addition to limited ability to generate active thyroid hormone as a pathophysiological mechanism in the generation of CH, the misfolded TGs may cause TG retention in the ER and premature degradation. It gives rise to a distention of ER, abnormality called as ER storage disease (ERSD) (Kim and Arvan, 1998). Misfolded proteins are degraded by the ER-associated degradation (ERAD) pathway. Howeaver, it is highly likely that hydrolysis of limited amounts of mutated TG molecules may escape from the ER and migrate to the colloid, allowing synthesis of thyroid hormone. This is a possible mechanism providing a minimum amount of thyroid hormones, as observed in most cases of defective TG synthesis.

The p.R277* mutation is the most frequently reported mutation in the *TG* gene in Caucasian population. This mutation occurs in a CpG rich region that is prone to C>T transversions due to deamination of 5-methylcytosine and its consequent replacement by thymine. The aforementioned mechanism provide an explanation for the relatively high frequency of this mutation. The CGA arginine codon is considered a hot spot for mutations in mammalian DNA. However, comparative analysis between the haplotypes segregating with the mutation p.R277* from three Argentinian families (Figure 3) suggests the possibility that this mutation was also derived from a common ancestral chromosome. The clinical heterogeneity, in particular concerning of CH and goiter development, in patients with p.R277* mutation has already been previously described (Abdul-Hassan et al., 2013, Brust et al., 2011; Caputo et al., 2007a; 2007b; Citterio et al., 2011; 2013 Gutnisky et al., 2004; Machiavelli et al., 2010; Nicholas et al., 2016; Pardo et al., 2009; Peteiro-Gonzalez et al, 2010; Rivolta et al., 2005; van de Graaf et al., 1999).

The missense mutations located in ACHE-like domain identified here (p.R2317Q, p.A2343P, and p.W2346R) may also cause TG retention in the ER and premature degradation. Until now, 11 missense mutations were reported to be present in the ACHE-like domain associated to CH, localized in exons 38, 40, 41, 44 and 47 (Table 4). Functional analysis suggests that the p.A2215D

mutation results in retention of the TG protein inside the ER and degradation via the proteasome system (Pardo et al., 2009), as already observed in the *cog/cog* congenital goiter mouse (Kim et al, 1998) and the *rdw/rdw* non-goitrous CH rat (Kim et al, 2000).

On the other hand, we present a cohort analysis to assess the nature and frequency of TG mutations in 48 patients from 31 families with CH due to a TG defects, including the 7 families analysed in the present work. The majority of them reported by our laboratory over the last 2 decades and previously published (Caputo et al., 2007a, 2007b; Caron et al., 2003; Citterio et al., 2011, 2013a, 2013b, 2015; Gutnisky et al., 2004; Machiavelli et al., 2010; Rivolta et al., 2005; Targovnik et al., 1993, 1995, 2001, 2010b, 2012). The baseline clinicopathologic characteristics and mutations are listed in Table 3. We demonstrate that in all these patients the low TG serum levels and elevated levels of serum TSH are associated with inactivating mutations in the TG gene. Mutations in both TG alleles were found in 27 families, for 9 families in homozygosity and 18 families were compound heterozygous. In four families (BA/BM, FM, G and M; Table 3) only one mutated allele was detected even after sequencing all exonic coding sequence, the promoter region, or the exon/intron boundaries of the TG gene. This can be considered as straightforward cases of haploid insufficiency in a context of recessive inheritance. In all four families the monoallelic mutation was a nonsense mutation, in two cases it was the p.R277* (families BA/BM and F), in another case it was p.R768* (family G) and in the remaining one it was p.E1835* (family M). Consequently, p.R277* is presented as homozygous, compound heterozygous or monoallelic variants. It is likely that the apparent absence of a second mutation could be explained by technical limitations of the direct sequencing analysis. Our analysis does not exclude micro deletions involving one or several exons or mutations in distant regulatory or intronic regions of the TG gene. Aditionally, we cannot disregard the possibility of oligogenicity in our monoallelic cases harboring additional mutations in other thyroid or non-thyroid genes that could contribute to the CH phenotype. The same situation

has previously been reported by Nicholas et al (2016), where they described several monoallelic TG mutations in individuals with mild CH and a severe hypothyroid patient harboring digenic pathogenic heterozygous variants in both TG and TPO genes, strongly indicating that the patient's disease was a consequence of oligogenicity.

The majority of the detected mutations occur in exons 4, 7, 38 and 40. 28 different mutations were identified, 33 of the 96 studied TG alleles were the p.R277* (Table 3). Ten patients from five families were homozygous for p.R277* mutation, other ten patients from seven families were compound heterozygous for the mutation p.R277*, whereas three cases from two families, as indicated above, were monoallelic for the TG defect.

In conclusion, in this work we have identified and characterized three novel mutations and four previously reported mutations in the TG gene in 8 patients from 7 non-consanguineous families and we have extended our analysis to a total of 31 families with TG defects identified in our laboratory. The identification and characterization of TG mutations that occur naturally in patients with thyroid dishormonogenesis is undoubtedly a valuable approach to study the TG structure/function relations. Aditionally, the identification of TG mutations as cause of CH also provides an important tool for clinical diagnosis and genetic counseling.

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Figure legends

Figure 1. Pedigrees showing the thyroglobulin genotype results for the families H, I, J, K, L, LL and M. Partial sequencing chromatograms of genomic DNA are shown. Squares represent males and circles females. Filled symbols denote affected individuals and half-filled symbols, unaffected heterozygous individuals. The hatched symbols indicate the c.378C>A [p.Y107*] and c.7084G>C [p.A2343P] mutated alleles and the solid symbols the c.886C>T [p.R277*], c.1351C>T [p.R432*] c.5560G>T [p.E1835*], c.7007G>A [p.R2317Q] and c.7093T>C [p.W2346R] mutated alleles. Sense strand is shown. Black arrows denote the position of identified mutations, single chromatogram peaks indicate homozygosity and two overlapping peaks at the same locus, heterozygosity. The DNA of the fathers from family L (L:I-1) and from family LL (LL:I-1), and the father and mother from family M (M:I-1 and M:1-2) were not available for analysis. Patient M:II-1 and M:II-2 from family M did not show a second inactivating mutation. Open arrows denote index patient. Novel inactivating mutations are highlighted in red.

Figure 2. Photographs, ultrasonographies, macroscopy and light microscopies of thyroid tissue from patient LL:II-1 of family LL. Panels a and b, clinical examination reveals a giant goiter with firm consistency in all the neck and supraclavicular hollow. Panels c (right lobe), d (isthmus) and e (left lobe), ultrasonographic studies evidenced a gland size augmented with multiple rounded echogenic images in both lobes, right lobe size: 114x51x52.4 mm, left lobe size: 109x48.3x49.6 mm and total volume: 294 ml. Panels f and g, macroscopic examination showed an excised thyroid with a weight of 229 g with irregular surface, predominantly solid light brown colour, with little cavities. Panels h, i, j and k, the histopathology aspect revealed follicles with signs of intense TSH stimulation, such as the epitelial hyperplasia, others with very much dilated lights of varied sizes

(indicated by an arrow) between follicles septum of connective tissue that clutter as nodules (hematoxylin-eosin staining; magnification, x100 and x400).

Figure 3. Comparative haplotype analysis of the patients A:III-2 (family A), B:II-2 (family B), K:II-1 (family K), RM and ME with the p.R277* mutation in homozygous state, using 15 exonic SNPs. The arrows denote differences between haplotypes. The parents of ME were not available for segregation analysis, consequently both haplotypes in this patient are hypothetical. Note that Patients A:III-2 (family A), B:II-2 (family B) and K:II-1 (family K) are homozygous for the same combinations of SNPs.

Figure 4. Schematic representation of the regions I, II and III, repetitive motifs, acetylcholinesterase homology domain and hormonogenic sites in the wild-type and putative mutant thyroglobulin proteins (p.Y107*, p.R277*, p.R432*, p.E1835*, p.R2317Q, p.A2343P and p.W2346R). The repetitive motifs (Types-1, 2 and 3) and the acetylcholinesterase homology domain (ACHE-like domain) are represented by boxes. The positions of T_4 (5, 1291 and 2747) and T_3 (2554) are shown. Novel inactivating mutations are highlighted in red.

Figure 5. Partial protein alignment of the Homo sapiens, Bos taurus, Rattus norvegicus, Mus musculus, Xenopus tropicalis, Dano rerio (Zebrafish) and Petromyzon marinus (Lamprey) TG species. Completely conserved residues are indicated in grey. The amino acids are indicated by the single-letter code and the positions of the missense mutations (p.R2317Q, p.A2343P and p.W2346R) are showed. The TG protein primary sequences are based on the NCBI reference sequence: Homo Sapiens (accession number: NP_003226.4), Bos Taurus (NP_776308.1), Rattus norvegicus (NP_112250.2), Mus musculus (NP_033401.2), Xenopus tropicalis (NP_001316486.1;

Holzer et al., 2016), Dano rerio (Zebrafish; NP_001316794; Holzer et al., 2016) and Petromyzon marinus (Lamprey; Holzer et al., 2016). Novel inactivating mutations are highlighted in red.

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Table 1. Biochemical and molecular data of the families with congenital hypothyroidism H, I, J, K, L, LL and M.

Families	Patients	Gender	Age at diagnosis	Serum TSH mIU/L	Serum TT_4 µg/dl	Serum FT_4 ng/dl	Serum TT_3 ng/dl	Serum FT_3 pg/dl	Serum TG ng/ml	Genomic change	TG genotype cDNA change	Protein change
H	H:II-2	F	35 ds	422 (0.85-7.9)	2.11 (7.3-17.7)	0.27 (1.01-2.09)	123 (142-366)	NA	<1 (1.3-100)	8:133898968C>T/ 8:134042113G>C	c.1351C>T/c.7084G>C	p.R432*/p.A2343P
I	I:II-3	F	18 ds	713. (1.15-7.61)	6.79 (7.3-16.5)	0.87 (1.11-1.96)	140 (124-315)	NA	1 (1.3-100)	8:133883696C>A/ 8:133894854C>T	c.378C>A/c.886C>T	p.Y107*/p.R277*
J	J:II-1	F	29 ds	72.1 (0.5-8)	4.2 (6-18)	0.68 (1-2.6)	168 (80-260)	NA	<0.9 (30-100)	8:133883696C>A/ 8:134034366G>A	c.378C>A/c.7007G>A	p.Y107*/p.R2317Q
K	K:II-1	M	14 ds	>100 (0.5-8)	NA	0.24 (1-2.6)	67 (80-260)	NA	<0.9 (30-100)	8:133894854C>T/ 8:133894854C>T	c.886C>T/c.886C>T	p.R277*/p.R277*
L	L:II-1	F	4.9 yrs re-evaluation at 14.7 yrs	>400 (0.5-6.4) >100 (0.5-6.4)	2.5 (6-14) 1.7 (6-14)	ND 0.20 (0.8-2.2)	75 (80-245) 29 (80-245)	NA NA	NA <1 (10-30)	8:133883696C>A/ 8:133894854C>T	c.378C>A/c.886C>T	p.Y107*/p.R277*
LL	LL:II-1	M	12.10 yrs	76.7 (0.5-5)	3.7 (6.09-12.23)	0.17 (0.58-1.24)	NA	NA	NA[d]	8:134042122T>C/ 8:134042122T>C	c.7093T>C/c.7093T>C	p.W2346R/p.W2346R
M	M:II-1 [a]	F	5ds	66 (0.60-6.82) [b]	NA	1 (0.62-1.93) [c]	NA	162 (142-545) [c]	undetectable	8:133978816G>T/ WT	c.5560G>T/WT	p.E1835*/WT
	M:II-2 [a]	F	3 ds	83.6 (0.94-9.65) [b]	NA	1.17 (0.85-1.93) [c]	NA	357 (142-480) [c]	NA [e]	8:133978816G>T/ WT	c.5560G>T/WT	p.E1835*/WT

Novel inactivating mutations are highlighted in red. The genomic position corresponds to the GRCh37 assembly. 8:, location in chromosome 8. The nucleotide position is designated according to TG mRNA reference sequence reported in National Center for Biotechnology Information (NCBI), accession number: NM_003235.4. The A of the ATG of the initiator methionine codon is denoted nucleotide +1. The amino acid positions are numbered after subtracting the 19-amino acid signal peptide. The laboratory testing reflects the hormonal situation before L-T4 substitution or after 1 month withdrawal (re-evaluation). Reference ranges are shown in brackets. M, male; F, female; NA, Not Available; yrs, years; ds, days; WT, wild type.

[a] Note that both patients, II-1 and II-2, of the family M received shortly before birth an intraamiotic injection of L-T_4.

[b] Reference range according to Lem et al. (2012); [c] reference range according to Soldin et al. (1995).

[d] Under LT_4 substitution the serum TG concentration was 0.2 ng/ml (reference range: 1,6-80), [e] cord TG undetectable.

Table 2. Thyroglobulin allele frequencies in genome Aggregation Database.

Ethnic group	Mutations		
	8:133894854C>T c.886C>T p.R277*	8:133898968C>T c.1351C>T p.R432*	8:134034366G>A c.7007G>A p.R2317Q
Ashkenazi Jewish	0.001084 (11/10,152)	0.000 (0/9,840)	0.000 (0/9850)
Latino	0.0006101 (21/34,420)	0.000 (0/33,574)	0.000 (0/33582)
European (non Finnish)	0.0004025 (51/126,718)	0.000008965 (1/111,548)	0.000 (1/111700)
European (Finnish)	0.00003877 (1/25,792)	0.000 (0/22,296)	0.000 (0/22300)
African	0.0002912 (7/24,038)	0.000 (0/15,282)	0.000 (0/15304)
South Asian	0.00003249 (1/30,782)	0.000 (0/30,776)	0.000 (0/30782)
East Asian	0.000 (0/18,866)	0.0001740 (3/17,238)	0.000 (0/17248)
Other	0.0006184 (4/6,468)	0.000 (0/5,486)	0.000 (0/5486)
Total	0.0003463 (96/277,236)	0.00001626 (4/246,040)	0.000004061 (1/246252)

The genomic, cDNA and protein changes are indicated. The genomic position corresponds to the GRCh37 assembly. 8:, location in chromosome 8. The Minor Allelic Frequency (MAF) in genome Aggregation Database (gnomAD) and the count of mutated TG alleles identified on the total TG alleles analyzed (in brackets) are given for each ethnic group. gnomAD available at http://gnomad.broadinstitute.org The c.378C>A [p.Y107*], c.5560G>T [p.E1835*], c.7084G>C [p.A2343P] and c.7093T>C [p.W2346R] mutations were not detected in gnomAD. The nucleotide position is designated according to TG mRNA reference sequence reported in National Center for Biotechnology Information (NCBI), accession number: NM_003235.4. The A of the ATG of the initiator methionine codon is denoted nucleotide +1. The amino acid positions are numbered after subtracting the 19-amino acid signal peptide.

Table 3. Summary of mutations in the thyroglobulin gene detected in families analyzed by our laboratory.

Families	Background	Patients	Gender	Serum TG ng/ml	Thyroid Size ml	TG genotype			References
						Genomic change	cDNA change	Protein change	
MA	Brazil	MA:II-1 MA	**M**	1.8 (0.5-18.0)	Large multinodular goiter, 178 g	8:133894854C>T/ 8:133935642C>T	c.886C>T/c.4588C>T	p.R277*/p.R1511*	Targovnik et al., 1993 Gutnisky et al., 2004
		MA:II-2 JNA	**M**	3.4 (0.5-18.0)	Large multinodular goiter, 203 g	8:133894854C>T/ 8:133935642C>T	c.886C>T/c.4588C>T	p.R277*/p.R1511*	
		MA:III-2 RSS	**M**	2.1 (0.5-18.0)	Diffuse goiter, 65 g	8:133894854C>T/ 8:133995594C>C	c.886C>T/c.6200-1G>C	p.R277*/ Skipping of exon 35	
HSN/AcSN	Brazil	Patient 1 HSN	**M**	<1	Multinodular goiter 35.2 g	8:133978943G>T/ 8:133978943G>T	c.5686+1G>T/c.5686+1G>T	Skipping of exon 30/ Skipping of exon 30	Targovnik et al., 1995 Targovnik et al., 2001
		Patient 2 AcSN	**F**	<1	Multinodular goiter 63 g	8:133978943G>T/ 8:133978943G>T	c.5686+1G>T/c.5686+1G>T	Skipping of exon 30/ Skipping of exon 30	
RAM/RMM	Argentina	Patient 1 RAM	**F**	3.9 (1-30)	Goiter 60 g	8:133894854C>T/ 8:133894854C>T	c.886C>T/c.886C>T	p.R277*/p.R277*	Rivolta et al., 2005
		Patient 2 RMM	**F**	<3.0 (1-30)	Goiter 65 g	8:133894854C>T/ 8:133894854C>T	c.886C>T/c.886C>T	p.R277*/p.R277*	
TD/PD	France	TD	**F**	<0.8	Fetal goitre	8:133898760delC/ 8:134030185G>A	c.1143delC/c.6725G>A	p.G362Gfs*21/p.R2223H	Caron et al.,2003
		PD	**M**	0.7	Fetal goitre	8:133898760delC/ 8:134030185G>A	c.1143delC/c.6725G>A	p.G362Gfs*21/p.R2223H	
GD	Argentina	GD	**F**	0.9 (2-30)	Small diffuse goitre	8:133885376G>A/ 133894727_ 133894728insA	c.548G>A/c.759_760insA	p.C164Y/p.L235Tfs*3	Caputo et al., 2007a
RS	Argentina	**RS**	**M**	3.3 (2-30)	Large non-nodular goitre	8:133894854C>T/ 8:134030161C>A	c.886C>T/c.6701C>A	p.R277*/p.A2215D	Caputo et al., 2007a
ME	Argentina	ME	**M**	0.9 (2-30)	Large goitre	8:133894854C>T/ 8:133894854C>T	c.886C>T/c.886C>T	p.R277*/p.R277*	Caputo et al., 2007a
LD	Argentina	LE	**M**	0.9 (2-30)	Diffuse goiter	8:133894854C>T/ 8:133935642C>T	c.886C>T/c.4588C>T	p.R277*/p.R1511*	Caputo et al., 2007b
		LD	**F**	NA	Small diffuse goiter	8:133894854C>T/ 8:133935642C>T	c.886C>T/c.4588C>T	p.R277*/p.R1511*	
PA	Argentina	**PA**	**F**	5.8 (11.5-98)	Small goitre	8:134030185G>A/ 8:134034365C>T	c.6725G>A/c.7006C>T	p.R2223H/p.R2317*	Machiavelli et al., 2010

PL/PC	Argentina	**PL**	**M**	1.4 (11.5-98)	Small goitre	8:134030161C>A/ 8:134030161C>A	c.6701C>A/c.6701C>A	p.A2215D/ p.A2215D	Machiavelli et al., 2010
		PC	**F**	6.9 (11.5-98)	Small goitre	8:134030161C>A/ 8:134030161C>A	c.6701C>A/c.6701C>A	p.A2215D/p.A2215D	
BA/BM	Argentina	**BA**	**M**	0.9 (2-30)	Moderate goitre.	8:133894854C>T/ WT	c.886C>T/WT	p.R277*/WT	Machiavelli et al., 2010
		BM	**F**	NA	Diffuse tender goitre	8:133894854C>T/ WT	c.886C>T/WT	p.R277*/WT	
FM	Argentina	**FM**	**M**	0.9 (2-30)	Large goitre	8:133894854C>T/ WT	c.886C>T/WT	p.R277*/WT	Machiavelli et al., 2010
A	Argentina	**A:III-1**	**M**	1 (1.40-78.50)	NA	8:133894854C>T/ 8:133894854C>T	c.886C>T/c.886C>T	p.R277*/p.R277*	**Citterio et al., 2013a**
		A:III-2	**M**	1 (1.40-78.50)	NA	8:133894854C>T/ 8:133894854C>T	c.886C>T/c.886C>T	p.R277*/p.R277*	
		A:III-3	**M**	1 (1.40-78.50)	3.6 (2.00)	8:133894854C>T/ 8:133894854C>T	c.886C>T/c.886C>T	p.R277*/p.R277*	
B	Argentina	**B:II-1**	**F**	NA	42 (11.6±4.4)	8:133894854C>T/ 8:133894854C>T	c.886C>T/c.886C>T	p.R277*/p.R277*	**Citterio et al., 2013a**
		B:II-2	**F**	1 (1.40-78.50)	271 (11.6±4.4)	8:133894854C>T/ 8:133894854C>T	c.886C>T/c.886C>T	p.R277*/p.R277*	
		B:II-3	**F**	1 (1.40-78.50)	151 (11.6±4.4)	8:133894854C>T/ 8:133894854C>T	c.886C>T/c.886C>T	p.R277*/p.R277*	
C	Argentina	**C:II-4**	**F**	2,9 (28.30-173)	7 (0.84±0.38)	8:133883696C>A/ 8:133919140G>A	c.378C>A/c.3842G>A	p.Y107*/p.C1262Y	**Citterio et al., 2013a**
D	Argentina	**D:II-3**	**F**	NA	NA	8:133900788delG/ 8:134030161C>A	c.2736delG/c.6701C>A	p.R893Rfs*54/p.A2215D	**Citterio et al., 2013a**
		D:II-4	**M**	1,27 (1.40-78.50)	6.4 (0.84±0.38)	8:133900788delG/ 8:134030161C>A	c.2736delG/c.6701C>A	p.R893Rfs*54/p.A2215D	
E	Argentina	**E:II-1**	**M**	1 (1.40-78.50)	9.6 (0.84±0.38)	8:133973317delA/ 8:134034365C>T	c.5466delA/c.7006C>T	p.K1803Kfs*30/p.R2317*	**Citterio et al., 2013a**
F	Argentina	**F:II-2**	**M**	1 (2.0-30.0)	9.7 (2.00)	8:133894854C>T/ 8:133984063C>G-8:134030065	c.886C>T/c.6000C>G-c.6605C>G	p.R277*/pC1981W-p.P2183R	**Citterio et al., 2013a**
G	UK	**G:II-1**	**M**	0.2 (<1)	NA	8:133900411C>T/ WT	c.2359C>T/WT	p.R768*/WT	**Citterio et al., 2013a**

		G:II-2	**F**	0,2 (<1)	Fetal goitre	8:133900411C>T/ WT	c.2359C>T/WT	p.R768*/WT	
H	Argentina	**H:II-2**	**F**	<1 (1.3-100)	4.82 (1.62±0.41)	8:133898968C>T/ 8:134042113G>C	c.1351C>T/c.7084G>C	p.R432*/p.A2343P	current study
I	Argentina	**I:II-3**	**F**	1 (1.3-100)	3.6 (1.62±0.41)	8:133883696C>A/ 8:133894854C>T	c.378C>A/c.886C>T	p.Y107*/p.R277*	current study
J	Argentina	**J:II-1**	**F**	<0.9 (30-100)	Goiter	8:133883696C>A/ 8:134034366G>A	c.378C>A/c.7007G>A	p.Y107*/p.R2317Q	current study
K	Argentina	**K:II-1**	**M**	<0.9 (30-100)	Goiter	8:133894854C>T/ 8:133894854C>T	c.886C>T/c.886C>T	p.R277*/p.R277*	current study
L	Argentina	**L:II-1**	**F**	NA	Soft diffuse goiter	8:133883696C>A/ 8:133894854C>T	c.378C>A/c.886C>T	p.Y107*/p.R277*	current study
LL	Argentina	**LL:II-1**	**M**	NA	294 (7.0±2.0)	8:134042122T>C/ 8:134042122T>C	c.7093T>C/c.7093T>C	p.W2346R/p.W2346R	current study
M	France	**M:II-1**	**F**	undetectable	5,7 (1.62±0.41)	8:133978816G>T/ WT	c.5560G>T/WT	p.E1835*/WT	current study
		M:II-2	**F**	NA	4.1 (1.62±0.41)	8:133978816G>T/ WT	c.5560G>T/WT	p.E1835*/WT	
Fr1	France	**Fr1:II-1**	**F**	undetectable	Goiter	8:133935642C>T/ 8:133961173C>T	c.4588C>T/c.5386C>T	p.R1511*/p.Q1777*	Targovnik et al., 2010b
Fr2	France	**Fr2:II-1**	**F**	0,3 (>30)	Fetal goitre	8:133894854C>T/ 8:133900258C>T	c.886C>T/c.2206C>T	p.R277*/p.Q717*	Citterio et al., 2011
Fr3	France	**Fr3:II-1**	**F**	<0.3 (<15)	Enlarged right lobe	8:133919086_ 133919087insT/ 8:133923781_ 133923782delAT	c.3788_3789insT/ c.4159+3_+4delAT	p.I1244Ifs*3/ Skipping of exon 19 or partially included by use of cryptic 5' splice site	Targovnik et al., 2012
Vi1	Vietnam	**Vi1:II-1**	**M**	<0.3 (<15)	23.2 (2.7±0.8)	8:133894215G>A/ 8:134034397T>A	c.745+1G>A/c.7036+2T>A	Skipping of exon 6 or partially included by use of cryptic 5' splice site/Skipping of exon 40	Citterio et al., 2015
Tu1	Turkey	**Tu1:II-1**	**M**	NA	Goiter	Proximal deletion: 8:134129940_ 134129966 Inversion: 8:134146928_ 134129967 Distal deletion: 8:134146929_ 134147746	Imperfect DNA inversion homozygote. DNA inversion of 16,962 bp from exon 48 to intron 45 in the TG gene associated with two deleted regions at both sides of the inversion limits. .		Citterio et al., 2013b
		Tu1:II-2	**M**	<0.9 (<15)	10				
		Tu1:II-4	**M**	<0.7 (<15)	Small goiter				

Novel inactivating mutations are highlighted in red. The genomic position corresponds to the GRCh37 assembly. 8:, location in chromosome 8. The nucleotide position is designated according to TG mRNA reference sequence reported in National Center for Biotechnology Information (NCBI), accession number: NM_003235.4. The A of the ATG of the initiator methionine codon is denoted nucleotide +1. The amino acid positions are numbered after subtracting the 19-amino acid signal peptide. intronic nucleotides located upstream of the exon have negative numbering, while those located downstream have positive numbering. Splicing mutations are annotated by using cDNA sequences. Frameshifting mutations are designated by “fs” after a description of the first amino acid affected by the nucleotide change (insertion or deletion) and the stop codon with “*”, followed by indication of the length of the shifted open reading frame from the first affected codon to the new stop codon. M, male; F, female; NA, Not Available. Reference ranges are shown in brackets.

Table 4. Nonsense Mutations and Missense Mutations in the ACHE-homology domain in the Thyroglobulin gene associated with congenital hypothyroidism.

Exon	Genomic change	cDNA change	Protein change	dbSNP rs id	gnomAD MAF	References
Nonsense mutations						
Exon 4	8:133883696C>A	c.378C>A	p.Y107*	NF	NF	Citterio et al., 2013
Exon 4	8:133883793C>T	c.475C>T	p.R140*	rs759267330	0.00003677 (9/244,788)	Nicholas et al., 2016
Exon 7	8:133894854C>T	c.886C>T	p.R277*	rs121912648	0.0003463 (96/277,236)	Abdul-Hassan et al., 2013, Brust et al., 2011; Caputo et al., 2007a; 2007b; Citterio et al., 2011; 2013 Gutnisky et al., 2004; Machiavelli et al., 2010; Nicholas et al., 2016; Pardo et al., 2009; Peteiro-Gonzalez et al, 2010; Rivolta et al., 2005; van de Graaf et al., 1999
Exon 9	8:133898968C>T	c.1351C>T	p.R432*	rs773142559	0.00001626 (4/246,040)	Kahara et al., 2012; Nicholas et al., 2016; Niu et al., 2009
Exon 9	8:133899200C>A	c.1583C>A	p.S509*	NF	NF	Nicholas et al., 2016
Exon 9	8:133899505C>T	c.1888C>T	p.Q611*	NF	NF	Cangul et al., 2014
Exon 9	8:133899528G>A	c.1911G>A	p.W618*	NF	NF	Cangul et al., 2014
Exon 9	8:133899580C>T	c.1963C>T	p.Q636*	rs771807370	0.0001340 (37/276,100)	Löf et al., 2016
Exon 9	8:133899748C>T	c.2131C>T	p.Q692*	rs778493270	0.000008202 (2/243,844)	Hishinuma et al., 2006
Exon 10	8:133900258C>T	c.2206C>T	p.Q717*	NF	NF	Citterio et al., 2011
Exon 10	8:133900363C>T	c.2311C>T	p.Q752*	rs778743706	0.00002889 (8/276,940)	Nicholas et al., 2016
Exon 10	8:133900411C>T	c.2359C>T	p.R768*	rs752966476	0.00001445 (4/276,878)	Agretti et al., 2013; Brust et al., 2011; Citterio et al., 2013
Exon 10	8:133900537C>T	c.2485C>T	p.Q810*	NF	NF	Narumi et al., 2011
Exon 13	8:133910427T>A	c.3153T>A	p.C1032*	NF	NF	Hu et al., 2016
Exon 20	8:133925447G>A	c.4310G>A	p.W1418*	NF	NF	Hishinuma et al., 2006
Exon 22	8:133935642C>T	c.4588C>T	p.R1511*	rs121912646	0.00009383 (26/277,102)	Caputo et al., 2007b; Gutnisky et al., 2004; Mendive et al., 2005; Targovnik et al., 1993; 2010
Exon 27	8:133961137C>T	c.5350C>T	p.Q1765*	NF	NF	Niu et al., 2009
Exon 27	8:133961173C>T	c.5386C>T	p.Q1777*	rs754658907	0.00002167 (6/276,832)	Targovnik et al., 2010

Exon 30	8:133978816G>T	c.5560G>T	p.E1835*	NF	NF	Current study
Exon 31	8:133980118C>A	c.5766C>A	p.Y1903*	NF	NF	Fu et al, 2016-1; Hu et al., 2016
Exon 37	8:134025928C>T	c.6481C>T	p.Q2142*	NF	NF	Pardo et al. 2009
Exon 40	8:134034365C>T	c.7006C>T	p.R2317*	rs144875913	0.00001624 (4/246,254)	Citterio et al., 2013; Liu et al., 2012; Machiavelli et al., 2010; Mittal et al., 2016
Exon 46	8:134144162C>T	c.7969C>T	p.Q2638*	NF	NF	Hishinuma et al., 2006
Exon 47	8:134145835C>T	c.8119C>T	p.R2688*	NF	0.000007217 (2/277,110)	Fu et al, 2016-1; Hu et al., 2016
Missense Mutations in the ACHE-homology domain						
Exon 38	8:134030161C>A	c.6701C>A	p.A2215D	rs370991693	0.00004470 (11/246,064)	Caputo et al., 2007a; Pardo et al. 2008; 2009; Machiavelli et al., 2010; Citterio et al., 2013
Exon 38	8:134030185G>A	c.6725G>A	p.R2223H	rs2069566	0.000008133 (2/245,916)	Caron et al., 2003; Machiavelli et al., 2010; Raef et al., 2010
Exon 40	8:134034315G>A	c.6956G>A	p.G2300D	NF	NF	Hishinuma et al., 2006
Exon 40	8:134034366G>A	c.7007G>A	p.R2317Q	rs121912650	0.000004061 (1/246,252)	Kitanaka et al., 2006 ; Hishinuma et al., 2006; Current study
Exon 41	8:134042113G>C	c.7084G>C	p.A2343P	NF	NF	Current study
Exon 41	8:134042122T>C	c.7093T>C	p.W2346R	NF	NF	Current study
Exon 41	8:134042150G>T	c.7121G>T	p.G2355V	NF	NF	Hishinuma et al., 2006
Exon 41	8:134042152G>A	c.7123G>A	p.G2356R	rs137854434	0.00002045 (5/244,528)	Hishinuma et al., 2005; 2006; Kanou et al.; 2007
Exon 44	8:134125733T>A	c.7640T>A	p.L2528Q	rs2979042	0.003976 (1102/277,162)	Nicholas et al., 2016
Exon 44	8:134125846C>T	c.7753C>T	p.R2566W	rs114211101	0.0004923 (136/276260)	Jiang et al., 2016 ; Hu et al., 2016
Exon 47	8:134145770G>T	c.8054G>T	p.W2666L	NF	NF	Nicholas et al., 2016

Novel inactivating mutations are highlighted in red. The genomic position corresponds to the GRCh37 assembly. 8:, location in chromosome 8. The nucleotide position is designated according to TG mRNA reference sequences reported in the National Center for Biotechnology Information (NCBI), accession number: NM_ 003235.4. The A of the ATG of the initiator methionine codon is denoted nucleotide +1. The amino acid positions are numbered after subtracting the 19-amino acid signal peptide. dbSNP, database of single nucleotide polymorphisms and multiple small-scale variations (available at https://www.ncbi.nlm.nih.gov/snp). MAF, Minor Allelic Frequency in genome Aggregation Database (gnomAD, available at http://gnomad.broadinstitute.org). The count of mutated TG alleles identified on the total TG alleles analyzed are given in brackets. NF, not found.

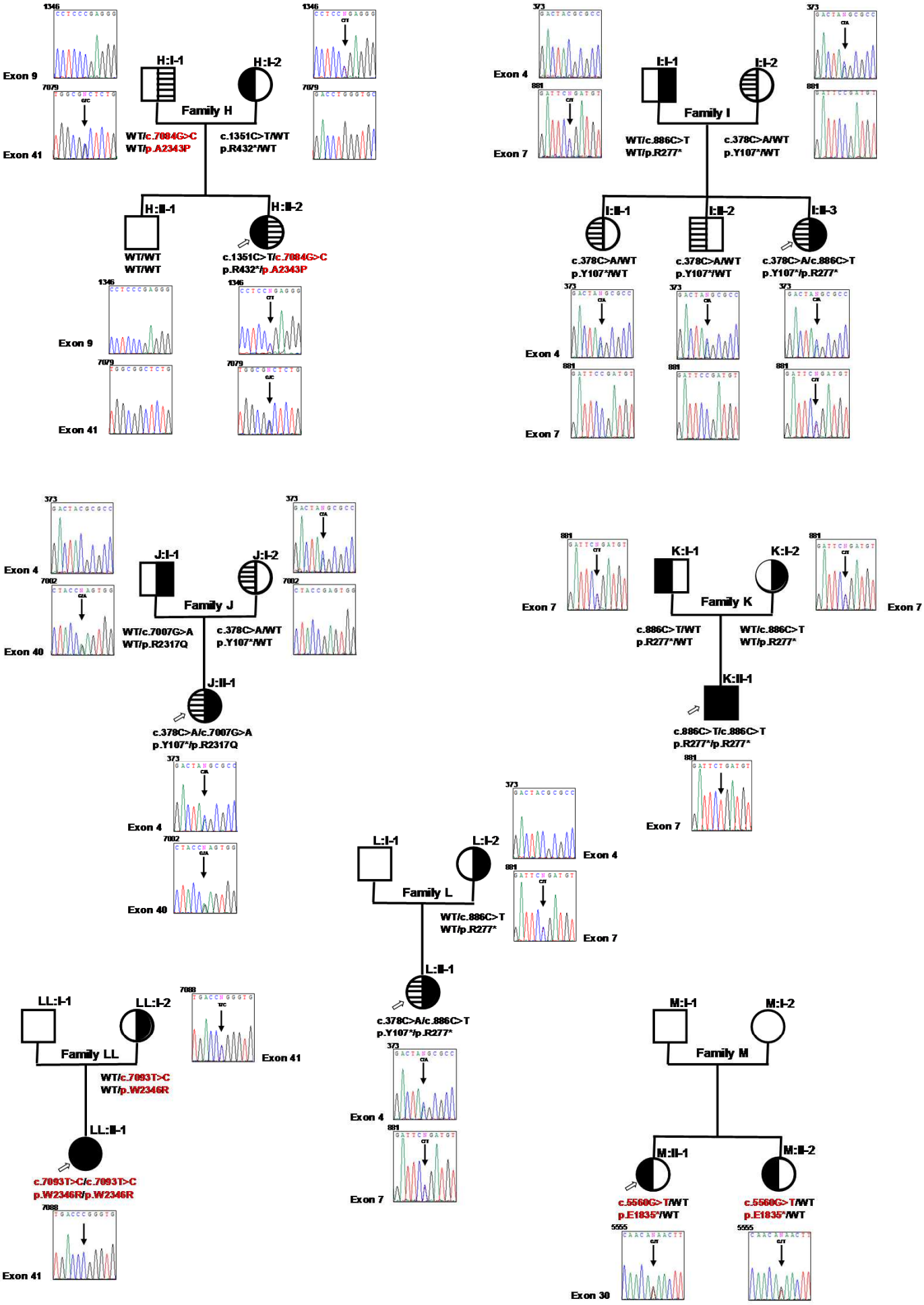
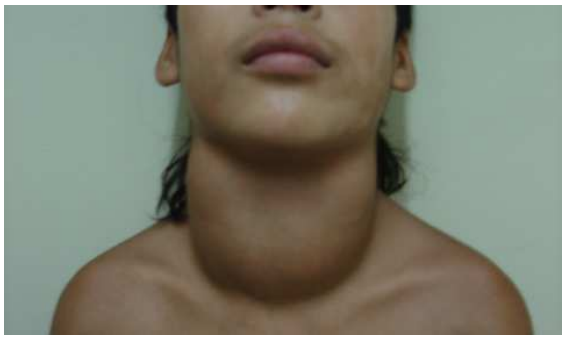
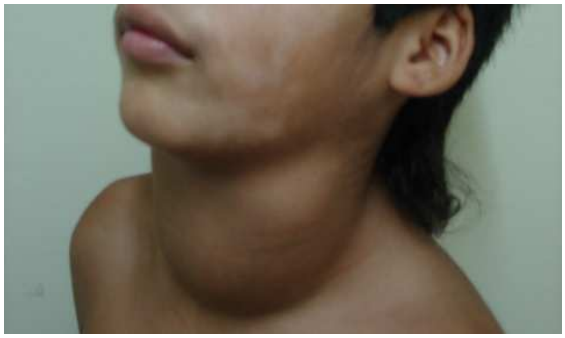

Exon 9
Exon 41
H:I-1
H:I-2
Family H
WT/c.7084G>C
WT/p.A2343P
c.1351C>T/WT
p.R432*/WT
H:II-1
H:II-2
WT/WT
WT/WT
c.1351C>T/c.7084G>C
p.R432*/p.A2343P
Exon 4
Exon 7
I:I-1
I:I-2
Family I
WT/c.886C>T
WT/p.R277*
c.378C>A/WT
p.Y107*/WT
I:II-1
I:II-2
I:II-3
c.378C>A/WT
p.Y107*/WT
c.378C>A/WT
p.Y107*/WT
c.378C>A/c.886C>T
p.Y107*/p.R277*
Exon 40
J:I-1
J:I-2
Family J
WT/c.7007G>A
WT/p.R2317Q
c.378C>A/WT
p.Y107*/WT
J:II-1
c.378C>A/c.7007G>A
p.Y107*/p.R2317Q
K:I-1
K:I-2
Family K
c.886C>T/WT
p.R277*/WT
WT/c.886C>T
WT/p.R277*
K:II-1
c.886C>T/c.886C>T
p.R277*/p.R277*
L:I-1
L:I-2
Family L
WT/c.886C>T
WT/p.R277*
L:II-1
c.378C>A/c.886C>T
p.Y107*/p.R277*
LL:I-1
LL:I-2
Family LL
WT/c.7093T>C
WT/p.W2346R
LL:II-1
c.7093T>C/c.7093T>C
p.W2346R/p.W2346R
M:I-1
M:I-2
Family M
M:II-1
M:II-2
c.5560G>T/WT
p.E1835*/WT
c.5560G>T/WT
p.E1835*/WT
Exon 30

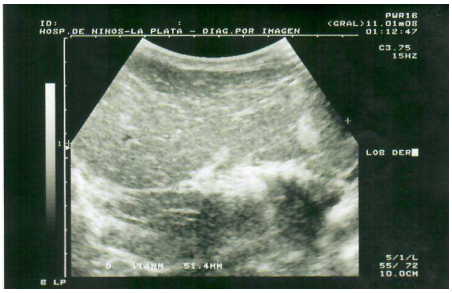
Figure 1



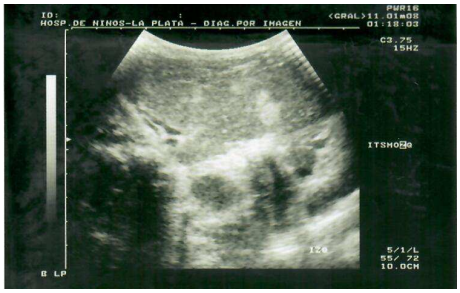
a



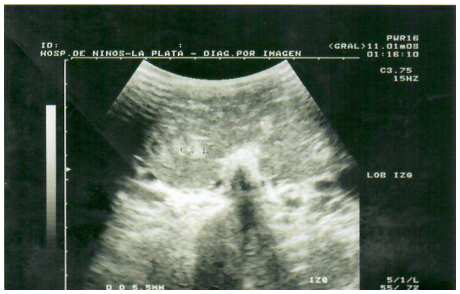
b



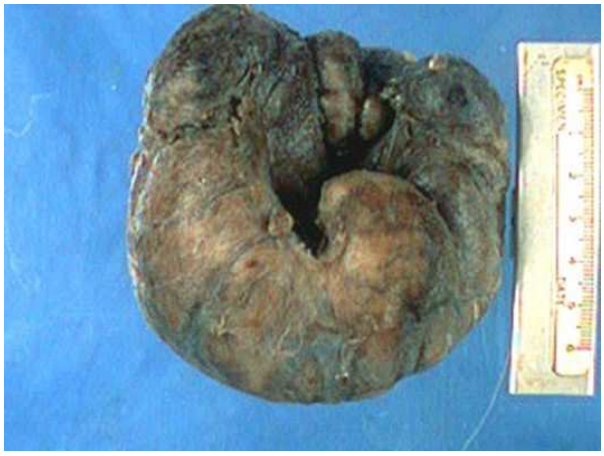
c



d



e



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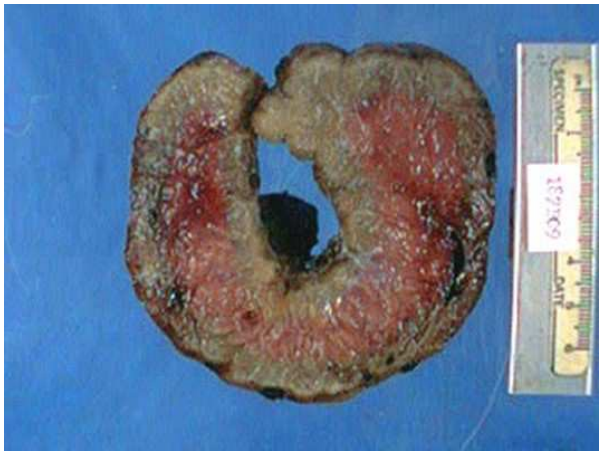
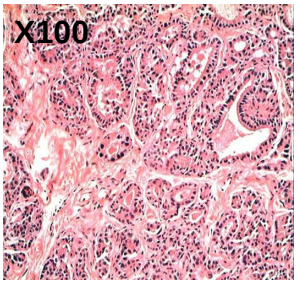
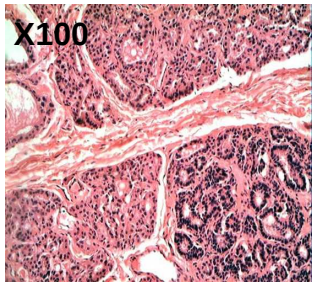


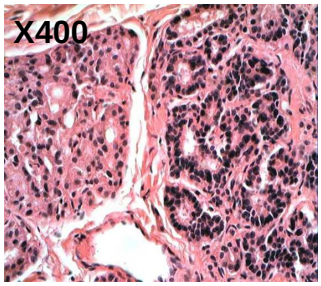
g



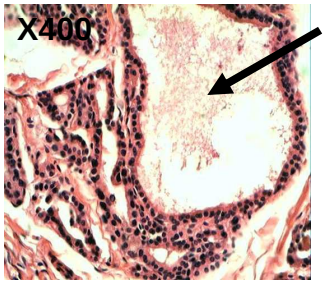
h



i



j



k

Figure 2

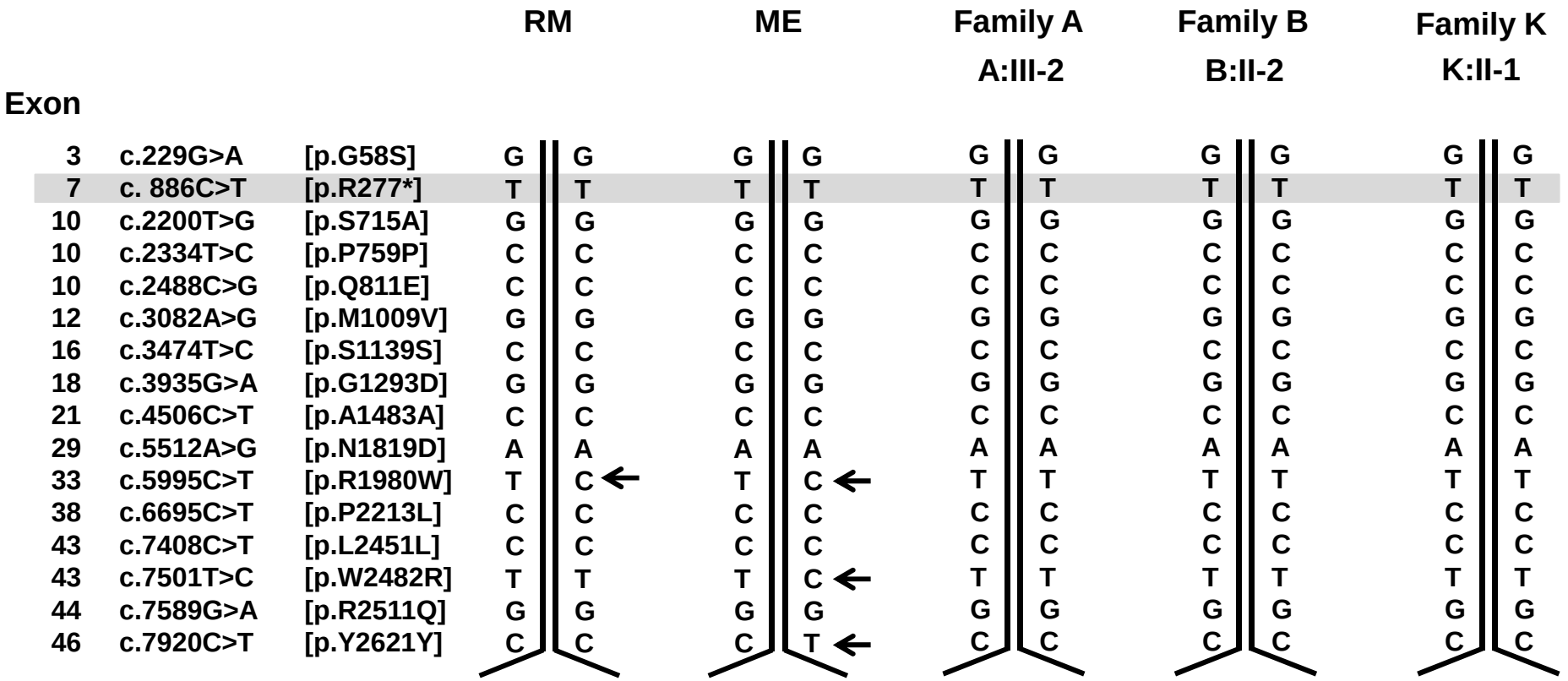


Figure 3

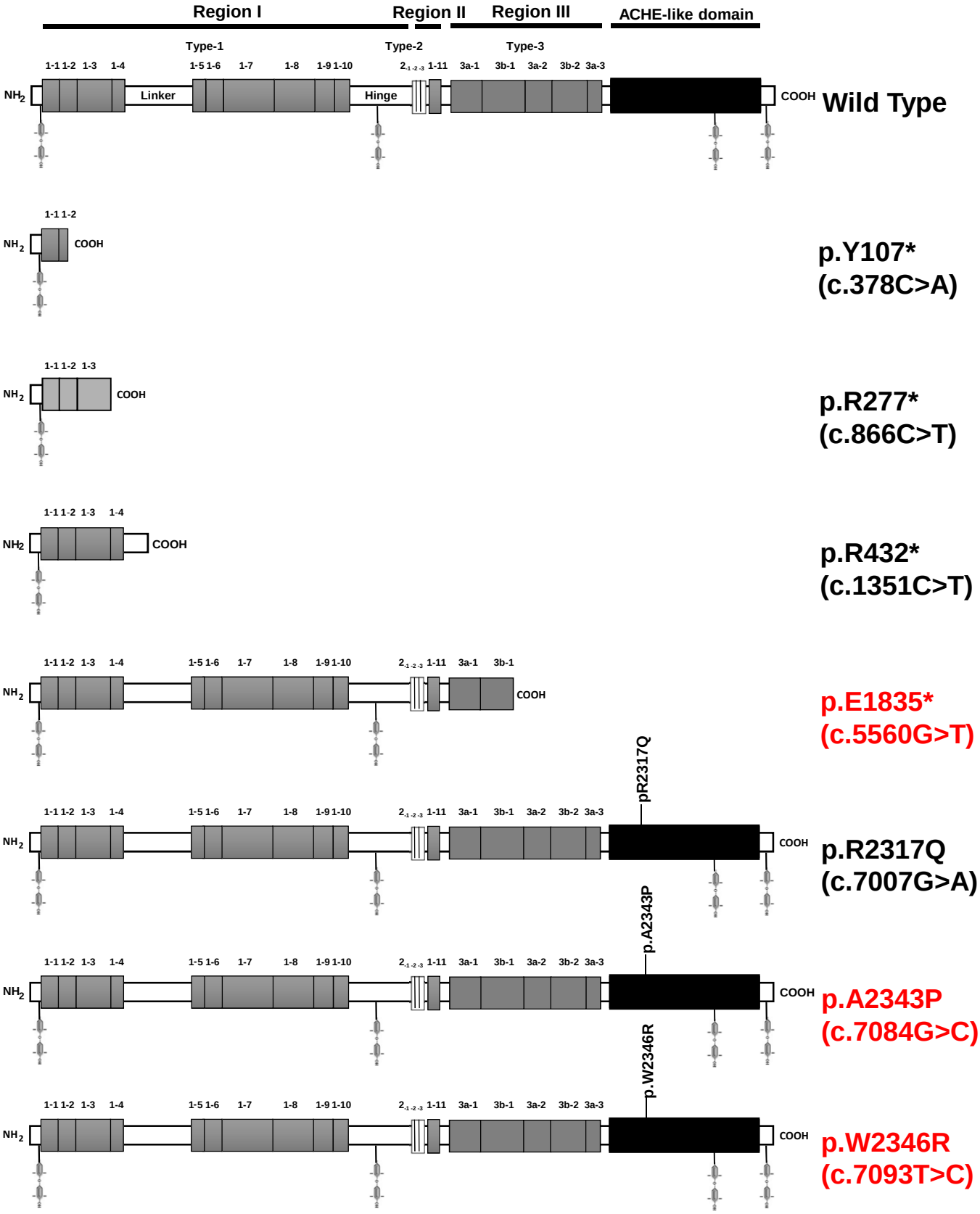

Region I
Region II
Region III
ACHE-like domain
Type-1
Type-2
Type-3
1-1 1-2 1-3 1-4 1-5 1-6 1-7 1-8 1-9 1-10 2-1 2-2 2-3 1-11 3a-1 3b-1 3a-2 3b-2 3a-3
NH2
Linker
Hinge
COOH
Wild Type
1-1 1-2
p.Y107*
(c.378C>A)
1-1 1-2 1-3
p.R277*
(c.866C>T)
1-1 1-2 1-3 1-4
p.R432*
(c.1351C>T)
1-1 1-2 1-3 1-4 1-5 1-6 1-7 1-8 1-9 1-10 2-1 2-2 2-3 1-11 3a-1 3b-1
p.E1835*
(c.5560G>T)
pR2317Q
1-1 1-2 1-3 1-4 1-5 1-6 1-7 1-8 1-9 1-10 2-1 2-2 2-3 1-11 3a-1 3b-1 3a-2 3b-2 3a-3
p.R2317Q
(c.7007G>A)
p.A2343P
p.A2343P
(c.7084G>C)
p.W2346R
p.W2346R
(c.7093T>C)

Figure 4

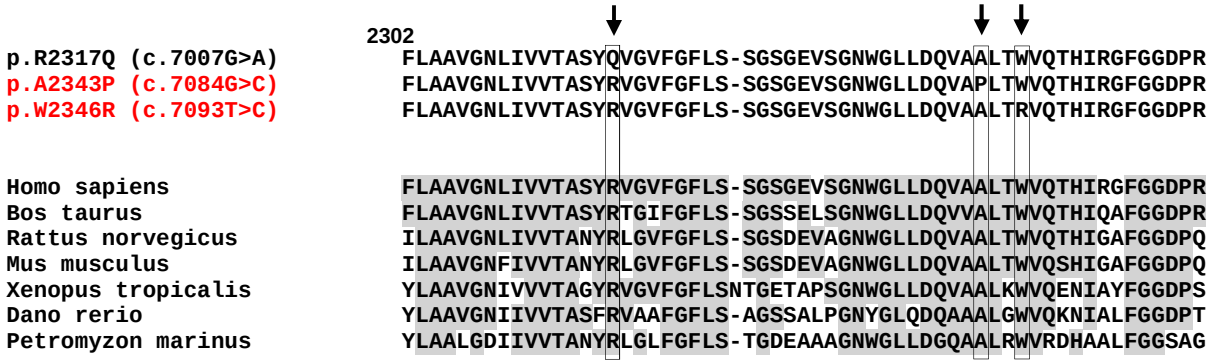

2302
p.R2317Q (c.7007G>A) FLAAVGNLIVVTASYQVGVFGFLS-SGSGEVSGNWGLLDQVAALTWVQTHIRGFGGDPR
p.A2343P (c.7084G>C) FLAAVGNLIVVTASYRVGVFGFLS-SGSGEVSGNWGLLDQVAPLTWVQTHIRGFGGDPR
p.W2346R (c.7093T>C) FLAAVGNLIVVTASYRVGVFGFLS-SGSGEVSGNWGLLDQVAALTRVQTHIRGFGGDPR
Homo sapiens FLAAVGNLIVVTASYRVGVFGFLS-SGSGEVSGNWGLLDQVAALTWVQTHIRGFGGDPR
Bos taurus FLAAVGNLIVVTASYRTGIFGFLS-SGSSELSGNWGLLDQVVALTWVQTHIQAFGGDPR
Rattus norvegicus ILAAVGNLIVVTANYRLGVFGFLS-SGSDEVAGNWGLLDQVAALTWVQTHIGAFGGDPQ
Mus musculus ILAAVGNFIVVTANYRLGVFGFLS-SGSDEVAGNWGLLDQVAALTWVQSHIGAFGGDPQ
Xenopus tropicalis YLAAVGNIVVVTAGYRVGVFGFLSNTGETAPSGNWGLLDQVAALKWVQENIAYFGGDPS
Dano rerio YLAAVGNIIVVTASFRVAAFGFLS-AGSSALPGNYGLQDQAAALGWVQKNIALFGGDPT
Petromyzon marinus YLAALGDIIVVTANYRLGLFGFLS-TGDEAAAGNWGLLDGQAALRWVRDHAALFGGSAG

Figure 5

Highlights

- We report eight patients with hypothyroidism due to thyroglobulin gene mutations.
- Molecular analysis reveals three novels and four previously reported mutations.
- Aditionally, we analyze a total of 31 unrelated families studied in our laboratory.
- 33 of the 96 studied TG alleles were the p.R277*.
- Our results confirm the genetic heterogeneity of thyroglobulin defects.