Recurrence of the p.R277X/p.R1511X compound heterozygous mutation in the thyroglobulin gene in unrelated families with congenital goiter and hypothyroidism: haplotype analysis using intragenic thyroglobulin polymorphisms

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Abstract

Thyroglobulin (TG) functions as the matrix for thyroid hormone synthesis. Thirty-five different loss-of-function mutations in the TG gene have been reported. These mutations are transmitted in an autosomal recessive mode. The objective of this study is to analyze the recurrence of the p.R277X/p.R1511X compound heterozygous mutation in the TG gene in two unrelated families (one Argentinian and another Brazilian) with congenital hypothyroidism, goiter and impairment of TG synthesis. The first and last exon of the TG gene, the exons where previously mutations and single nucleotide polymorphisms (SNPs) were detected, as well as the TG promoter, were analyzed by automatic sequencing in one affected member of the each family. Four microsatellite markers localized in introns 10, 27, 29 and 30 of the TG gene, one insertion/deletion intragenic polymorphism and 15 exonic SNPs were used for haplotype analysis. A p.R277X/p.R1511 compound heterozygous mutation in the TG gene was found in two members of an Argentinian family. The same mutations had been also reported previously in two members of a Brazilian family. We constructed mutation-associated haplotypes by genotyping members of the two families. Our results suggest that the cosegregating haplotype is different in each one of these families. Different haplotypes segregated with the p.R277X and p.R1511 mutations demonstrating the absence of a founder effect for these mutations between Argentinian and Brazilian populations. However, haplotyping of Argentinian patients showed the possibility that the p.R277X alleles might be derived from a common ancestral chromosome.

Journal of Endocrinology (2007) **195**, 167–177

Introduction

Synthesis of tri-iodothyronine (T_3) and thyroxine (T_4) follows a metabolic pathway that depends on the integrity of the thyroglobulin (TG) structure (Rivolta & Targovnik 2006). This large glycoprotein is a homodimer of 2749 residues synthesized and secreted by the thyroid cells into the lumen of thyroid follicle. In humans, it is coded by a single copy gene, 270 kb long (GenBank accession number NT_008046), that maps on chromosome 8q24 and contains an 8·5 kb coding sequence (GenBank accession no NM_003235) divided into 48 exons (Malthièry & Lissitzky 1987, van de Graaf *et al.* 1997, 2001, Mendive *et al.* 2001, Rivolta & Targovnik 2006).

In the two last decades, a series of mutations in the TG gene were reported. Four of them have been observed in Afrikander cattle (p.R697X; Ricketts *et al.* 1987), Dutch goats (p.Y296X; Veenboer & de Vijlder 1993), cog/cog mouse (p.L2263P; Kim *et al.* 1998), and rdw rats (p.G2300R; Hishinuma *et al.* 2000, Kim *et al.* 2000). Mutations in the human TG gene are associated with congenital goiter (Ieiri *et al.* 1991, Targovnik *et al.* 1993, 1995, 2001, Medeiros-Neto *et al.* 1996, van de Graaf *et al.* 1999, Hishinuma *et al.* 1999, 2005, 2006, Caron *et al.* 2003, Gutnisky *et al.* 2004, Mendive *et al.* 2005, Rivolta *et al.* 2005, Alzahrani *et al.* 2006, Kitanaka *et al.* 2006) or endemic (Pérez-Centeno *et al.* 1996) and non-endemic simple goiter (Corral *et al.* 1993, González-Sarmiento *et al.* 2001). Thirty-five inactivating mutations have been identified and characterized in the human TG gene: 20 missense mutations (p.C175G, p.Q310P, p.Q851H, p.S971I, p.R989C, p.P993L, p.C1058R, p.C1245R, p.S1447N, p.C1588F, p.C1878Y, p.I1912V, p.C1977S, p.C1987Y, p.C2135Y, p.R2223H, p.G2300D, p.R2317Q, p.G2355V, p.G2356R), 5 nonsense mutations (p.R277X, p.Q692X, p.W1418X, p.R1511X, p.Q2638X), 8 splice site mutations (g.IVS3-3C > G, g.IVS5 + 1G > A,

0022–0795/07/0195–167
DOI: 10.1677/JOE-07-0033
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g.IVS10-1G>A, g.IVS24+1G>C, g.IVS30+1G>T, g.IVS30+ lG>A, g.IVS34-1G>C, g.IVS45+2T>A) and 2 single nucleotide deletions (p.G362fsX382, p.D1494fsX1547) (Ieiri *et al.* 1991, Corral *et al.* 1993, Targovnik *et al.* 1993, 1995, 2001, Medeiros-Neto *et al.* 1996, Pérez-Centeno *et al.* 1996, van de Graaf *et al.* 1999, Hishinuma *et al.* 1999, 2005, 2006, González-Sarmiento *et al.* 2001, Caron *et al.* 2003, Gutnisky *et al.* 2004, Mendive *et al.* 2005, Rivolta *et al.* 2005, Alzahrani *et al.* 2006, Kitanaka *et al.* 2006). Rivolta *et al.* (2005) reported that the p.R277X mutation is the most frequently identified TG mutation in south American population and haplotype studies suggest that a mutational hot spot could explain the recurrence of this mutation. In contrast, haplotype analysis in euthyroid to mildly hypothyroid Japanese patients reveals a founder effect of the frequently found mutations p.C1058R and p.C1977S (Hishinuma *et al.* 2006). Interestingly, in this population, patients with p.C1245R mutation possessed different haplotypes from other patients with the same mutation, suggesting that some of the C1245R alleles were due to independently recurrent mutations (Hishinuma *et al.* 2006). An alternative explanation is that p.C1245R is an old mutation with new single nucleotide polymorphisms (SNPs) being created independently.

We have previously identified a p.R277X/p.R1511X compound heterozygous TG mutation in two members of a Brazilian family with a complex history of congenital goiter (Targovnik *et al.* 1993, Gutnisky *et al.* 2004, Mendive *et al.* 2005).

We report here a new case of congenital goitrous hypothyroidism in an Argentinian family, which is a compound heterozygous for the p.R277X/p.R1511X mutations. In order to evaluate whether these mutations were inherited from a common ancestral chromosome or whether they are independent recurrent mutations in heterogeneous genetic backgrounds, we studied 20 polymorphic markers within the TG gene in one affected member of each family. The haplotype studies suggest the absence of a founder effect for the p.R1511X and p.R277X mutations between Argentinian and Brazilian populations. In contrast, comparative analysis between the haplotypes segregating with the mutation p.R277X from two Argentinian families suggests the possibility that this mutation was derived from a common ancestral chromosome.

Materials and Methods

Clinical report

LD Family

Index LD patient. The patient is the third child of a nonconsanguineous couple referred for a high thyroid-stimulating hormone (TSH) level in the neonatal screening at day 3 of life. She was born at term after a noncomplicated pregnancy and delivery. Her birth weight was 3650 g and in the first visit she looked pale with slight jaundice. Hypothyroidism was confirmed and treatment with L-thyroxin started. At age 3, still under treatment, a soft and small goiter was palpated. At age 5, treatment was suspended for a month and she was reevaluated. Her thyroid scan showed a diffuse goiter, TG serum levels were undetectable and the perchlorate discharge absent. A defect in TG synthesis was suspected. Treatment was reinitiated and she grew and developed normally.

LE Patient. This boy is the first child of this family. He was 11 years old when the hypothyroidism was diagnosticated in the sister (LD). He was born from an uneventful pregnancy and delivery with birth weight 4020 g. At age 3, a goiter was noticed and he was treated with hormone replacement. When he was seen for the first time at the Division of Endocrinology he was a normal boy, in Tanner stage 2, receiving L-thyroxin and he had a diffuse goitre. Thyroid function before treatment showed high TSH levels, low T_4 and normal T_3. Treatment was adjusted and the goiter reduced its size. His follow up was discontinuous, but he grew and developed normally.

The results of thyroid function tests of the LD family members studied are shown in Table 1.

The parents and a healthy sister of this family were evaluated. Thyroid function was normal in all of them, without thyroid autoimmunity.

MA Family This family was extensively studied by Targovnik *et al.* (1993), Gutnisky *et al.* (2004), Mendive *et al.* (2005). In brief, two affected siblings and one of their nephews had congenital goiter, hypothyroidism, and marked impairment of

Table 1 Thyroid function tests from relatives and patients of LD family with congenital goitrous hypothyroidism and impairment of thyroglobulin synthesis

Subjects	Serum TSH (mU/l)	Serum TT_4 (nmol/l)	Serum TT_3 (nmol/l)	Serum TG (μg/l)	Anti-TPO antibodies (IU/ml)	Anti-TG antibodies (IU/ml)
LA (father)	3·8	115·2	2·4	ND	<10	ND
LG (mother)	1·5	99·0	1·8	ND	<10	<20
LE[a]	>60	60·4	3·0	0·9	<10	<20
LM	1·1	146·7	3·0	ND	<10	ND
LD[a]	>50	47·6	2·8	ND	<10	<20
Normal range	<5	77–180	1·22–3·07	2–30	<20	<20

Conversion to conventional units: TT_4, nmol/l ÷ 12·87 μg/dl; TT_3, nmol/l ÷ 0·01536 ng/dl. ND, not determined.
[a]Affected individuals with thyroglobulin defect.

TG synthesis (Targovnik *et al.* 1993, Gutnisky *et al.* 2004, Mendive *et al.* 2005). Molecular analysis showed that the two affected siblings are compound heterozygous for p.R277X/p.R1511X in the TG gene. Segregation studies indicate that their mother and one unaffected sister are heterozygous for the p.R277X mutation, whereas their father and three unaffected brothers are all heterozygous for the p.R1511X mutation (Gutnisky *et al.* 2004). In addition, the symptomatic nephews have inherited one copy of the p.R277X from their mother and one copy of the g.IVS34-1G>C mutation from their father (Gutnisky *et al.* 2004).

RM Patient This Argentinian patient with congenital goiter and hypothyroidism was extensively investigated by us from clinical, biochemical, and molecular biology standpoints (Targovnik *et al.* 1990, Rivolta *et al.* 2005). Laboratory tests demonstrated low serum total T_4 (TT_4) and T_3 (TT_3) concentrations, lower normal limit serum TG, and elevated serum TSH values. Molecular analysis revealed a homozygous p.R277X mutation in the TG gene. The parents of RM were not available for segregation analysis.

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

Thyroid function tests

TT_4, TT_3, and serum TSH levels were determined by ECLIA ELECSYS system (Roche). Serum TG concentration was measured using IFMA Delfia (Perkin–Elmer, Turku, Finland). Anti-TPO and anti-TG antibodies were determined by ICMA Immulite (Diagnostic Products Corporation, Los Angeles, CA, USA).

Genomic DNA isolation

Genomic DNA was isolated from peripheral blood leucocytes by the standard cetyltrimethylammonium bromide method (Murray & Thompson 1980).

DNA sequencing

The 180 bp of the promotor region and the exons 1, 3, 4, 5, 7, 9, 10, 12, 16, 17, 18, 21, 22, 29, 30, 33, 35, 38, 43, 44, 46, and 48 of the human TG gene, including splicing signals and the flanking intronic regions of each intron, were amplified using the primers and PCR conditions reported previously (Caron *et al.* 2003, Gutnisky *et al.* 2004). Both the sense and antisense strands were sequenced using the same TG-specific primers used in the amplification with the Big Dyedeoxyterminator Cycle Sequencing Kit (Applied Biosystems, Weiterstadt, Germany). The samples were analyzed on the ABI Prism 3100 DNA sequencer (Applied Biosystems).

Microsatellite genotyping

The Tgms1, Tgms2, TGrI29, and TgrI30 microsatellites, localized in introns 10, 27, 29, and 30 of the human TG gene respectively, were typed as reported elsewhere (Rivolta *et al.* 2002, Tomer *et al.* 2002). PCR products were resolved by electrophoresis in 6% polyacrylamide denaturing gels.

The allele sizes were easily determined by comparison with the M13 mp18 sequences. In addition to the main amplification product, each allele presents a typical shadow product that is 2 (Tgms1, Tgms2, TGrI29) or 4 bp (TGrI30) smaller and less intense. The reason may be slippage during PCR amplification or incomplete extension by the polymerase (Rivolta *et al.* 2002).

IndelTG-IVS18 polymorphism genotyping

The large insertion/deletion (indel) polymorphism of 1464 bp (IndelTG-IVS18) localized in intron 18 of the human TG gene was analyzed by multiplex PCR (Moya *et al.* 2003, Gutnisky *et al.* 2004), using the primers and PCR conditions described previously. The amplified fragments were analyzed in a 2% agarose gel. The amplification generates two fragments of 374 and 541 bp, indicating the exclusion or inclusion of the indel polymorphic region respectively.

c.4506 C>T SNP genotyping

MspI endonuclease was used to screen for the presence of the 4506 C>T SNP in exon 21 (p.A1483A). The primers and PCR conditions for amplification of the exon 21 were described previously (Caron *et al.* 2003, Gutnisky *et al.* 2004). M13 sequences (18 nucleotides long) have been incorporated at the 5′ end of the forward and reverse primers. The amplified products (264 bp, 228 of them are TG sequences) were cleaved with *MspI* restriction endonuclease according to the specifications of the manufacturer (New Englans Biolabs, Ipswich, MA, USA) and analyzed by electrophoresis in 2% agarose gel. The homozygous C form showed fragments of 149 and 115 bp, whereas the homozygous T form maintained the 264 bp amplified fragment.

c.7589G>A SNP genotyping

TaqI endonuclease was used to screen for the presence of the c.7589G>A SNP in exon 44 (p.R2511Q). The primers and PCR conditions were described previously (Mendive *et al.* 1997, Gutnisky *et al.* 2004). Non TG-specific sequences (ten nucleotides long) have been incorporated at the 5′ end of the forward and reverse primers. The samples were cleaved with TaqI restriction endonuclease according to the specifications of the manufacturer (Fermentas Inc., Hanover, MD, USA) and analyzed by electrophoresis in 12% polyacrylamide gel. The amplified products (201 bp, 181 of them are TG sequences) contain two TaqI sites (positions 7587 polymorphic and 7667 not polymorphic). The TaqI restriction showed three fragments (27, 80, 94 bp) in the G homozygous form and two fragments (94 and 107 bp) in the A homozygous form.

Identification of 886C>T mutation by AlwN I restriction analysis

The mutation detected at position 886 in exon 7 created an AlwN I recognition site (van de Graaf *et al.* 1999). A 252 bp fragment containing exon 7 was generated by PCR under conditions described previously (Caron *et al.* 2003, Gutnisky *et al.* 2004), using the same intronic forward and reverse exon 7 primers. The forward and reverse primers contain M13 sequences (18 nucleotides long). Restriction enzyme digestion with AlwN I was performed as recommended by the manufacturer (New England Biolabs). After digestion, the DNA fragments were separated on a 2·5% agarose gel. Digestion of the mutant allele resulted in two fragments of 199 and 53 bp.

Identification of 4588C>T mutation by TaqI restriction analysis

The mutation detected at position 4588 in exon 22 destroys a TaqI recognition site (Targovnik *et al.* 1993, Gutnisky *et al.* 2004). A 316 bp fragment containing exon 22 was generated by PCR, under identical PCR conditions as described previously (Caron *et al.* 2003, Gutnisky *et al.* 2004), using the intronic forward and reverse exon 22 primers. The forward and reverse primers contain M13 sequences (18 nucleotides long). Restriction enzyme digestion with TaqI was performed as recommended by the manufacturer (Fermentas Inc). After digestion, the DNA fragments were separated on a 2·5% agarose gel. Digestion of the wild-type allele results in two fragments of 125 and 191 bp.

The nucleotide and amino acid nomenclatures

The nucleotide position in human TG mRNA is designated according to reference sequences (GenBank accession no NM_003235). The A of the ATG of the initiator methionine codon is denoted as nucleotide +1. The amino acid positions in Afrikander cattle, Dutch goats, cog/cog mouse, rdw rats, and human TG proteins are numbered after subtracting the amino acid signal peptide.

Results

DNA sequence analysis of the TG gene

The first and last exons of the TG gene, the exons and their intronic flanking regions where previously mutations and polymorphisms were detected, as well as the TG promoter, were analyzed from the index LD patient of the LD family. The GT–AG splicing consensus sequences were rigorously

Table 2 Single nucleotide polymorphisms (SNPs) frequencies by direct sequencing from the 20 alleles of the human thyroglobulin gene

	229G/A p.G58S	**2200T/G p.S715A**	**2488C/G p.Q811E**	**3082A/G p.M1009V**	**3935G/A p.G1293D**	**5512A/G p.N1819D**	**5995C/T p.R1980W**	**6695C/G p.P2213L**	**7501T/C p.W2482R**	**7589G/A p.R2511Q**
SNPs with amino acid change										
Allele 1	G: 19	T: 5	C: 20	A: 6	G: 18	A: 14	C: 7	C: 20	T: 9	G: 17
Allele 2	A: 1	G: 15	G: 0	G: 14	A: 2	G: 6	T: 13	G: 0	C: 11	A: 3

	2334T/C p.P759P	**3474T/C p.S1139S**	**4506C/T p.A1483A**	**7408C/T p.L2451L**	**7920C/T p.Y2621Y**
SNPs without amino acid change					
Allele 1	T: 6	T: 0	C: 15	C: 17	C: 10
Allele 2	C: 14	C: 20	T: 5	T: 3	T: 10

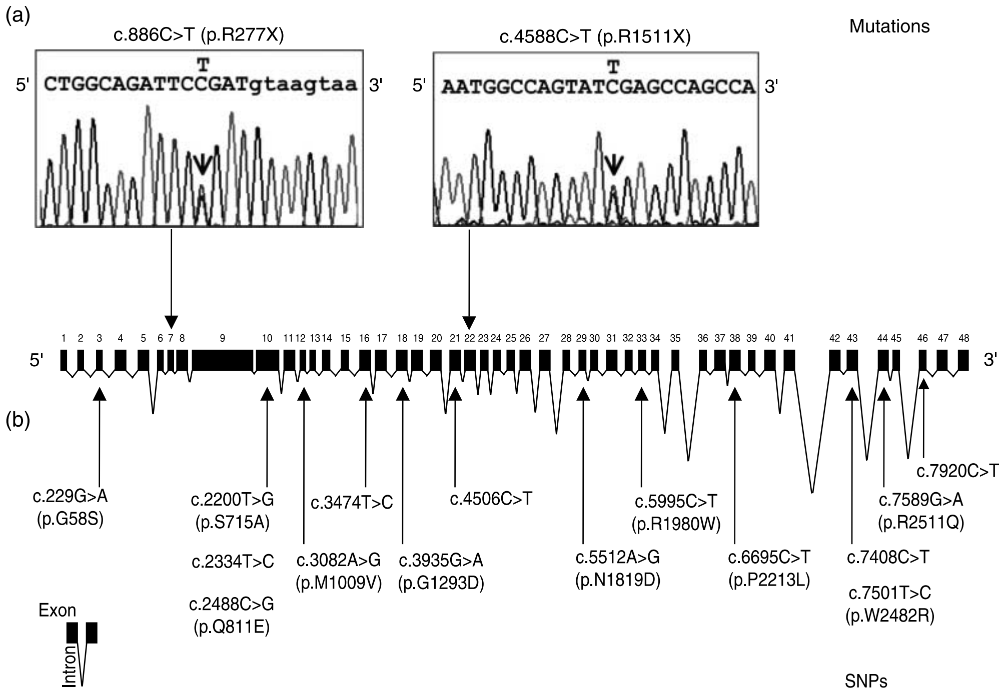

Figure 1 Identification of a p.R277X/p.R1511X heterozygous compound mutation in the index LD patient and schematic respresentation of the exon/intron organization of the thyroglobulin gene with indication of positions of the exonic single nucleotide polymorphisms (SNPs). (a) (left) Automated fluorescence-based direct sequencing of the exon 7 PCR fragment demonstrated a cytosine to thymine transition heterozygous at nucleotide position 886 (c.886C>T), which results in a termination codon at position 277 (p.R277X). (right) Automated fluorescence-based direct sequencing of the exon 22 PCR fragment demonstrated a cytosine to thymine transition heterozygous at nucleotide position 4588 (c.4588C>T), which results in a termination codon at position 1511 (p.R1511X). (b) The SNPs at the nucleotide levels are described denoting the A of the initiator ATG as nucleotide +1. The amino acid positions in nonsynonymous substitutions are numbered after subtracting the 19 amino acid signal peptide. Note the difference between scales used for introns and exons.

respected in all introns analyzed. Direct sequencing revealed a cytosine to thymine transition at nucleotide position 886 (c.886C>T) in exon 7 which results in a termination codon at position 277 (p.R277X) (Fig. 1) and also a cytosine to thymine transition at nucleotide 4588 (c.4588C>T) in exon 22 that results in a termination codon at position 1511 (p.R1511X) (Fig. 1). This finding established the compound heterozygous inheritance of the defect (p.R277X/p.R1511X). Previous reports indicate that skipping of the mutated exon 22 in the pre-mRNA restores the normal reading frame disrupted by the p.R1511X mutation (Targovnik *et al.* 1993, Gutnisky *et al.* 2004).

Haplotype frequency analysis of the SNP, microsatellites, and indel TG markers

In order to update the population frequencies of the polymorphisms of the TG gene, we partially repeated and extended our earlier studies. We analyzed the haplotype frequencies of the 15 exonic TG SNPs (van de Graaf *et al.* 1997, 2001, Mendive *et al.* 1997, Hishinuma *et al.* 1999, 2006, Rivolta & Targovnik 2006), the microsatellites located in introns 10 (Tgms1), 27 (Tgms2), 29 (TGrI29), and 30 (TGrI30) (Rivolta & Targovnik 2002, Tomer *et al.* 2002) and the previously characterized Indel (Moya *et al.* 2003) located in intron 18 (IndelIVSTG18).

We sequenced the exons 3, 10, 12, 16, 18, 21, 29, 33, 38, 43, 45 and 46 of the TG gene from ten unrelated individuals. SNPs exon position is indicated in Fig. 1*b* and the allele frequencies are summarized in Table 2.

The genotyping were carried out for the Tgms1, Tgms2, TGrI29, and TGrI30 microsatellites in a population sample of 100 unrelated individuals. The allele frequencies, heterozygosity index, and polymorphism information content (PIC) are summarized in Table 3. The Tgms1 showed a negative PIC and should be used only in association analysis, whereas Tgms2, TGrI29, and TGrI30 were informative polymorphic markers and well suited for linkage and association studies (Fig. 2).

From the 100 samples analyzed for IndelIVSTG18 genotyping, 36 were homozygous for the allele with the insertion, 18 were homozygous for the allele with the deletion, and 46 were heterozygous, giving allele frequencies of 0·59 (insertion) and 0·41 (deletion).

Table 3 Summary of measures of variation in Tgms1, Tgms2, TGrI29, and TGrI30 microsatellites

	Location	Allele	Allele frequencies	HET	PIC
STR					
Tgms1	Intron 10	305	0·460	0·761	−0·009
		307	0·530		
		309	0·010		
Tgms2	Intron 27	320	0·015	0·846	0·776
		322	0·010		
		324	0·005		
		326	0·260		
		328	0·020		
		330	0·020		
		332	0·005		
		336	0·005		
		338	0·340		
		340	0·070		
		342	0·090		
		344	0·015		
		346	0·110		
		348	0·030		
		350	0·005		
TGrI29	Intron 29	197	0·270	0·855	0·355
		199	0·245		
		201	0·475		
		203	0·010		
TGrI30	Intron 30	502	0·150	0·558	0·367
		506	0·075		
		510	0·005		
		530	0·010		
		534	0·055		
		538	0·700		
		542	0·005		

HET, heterozygosity; PIC, polymorphism information content.

Segregation analysis of the mutations in TG gene

Analysis by direct sequencing of PCR products of exons 7 and 22 from each member of the family showed that both siblings affected with goiter and hypothyroidism, LD and LE, have inherited one copy of the p.R277X mutation from their father, and one copy of the p.R1511X mutation from their mother. No mutations were found in the unaffected LM sister.

The exonic TG SNPs c.4506C>T (using MspI restriction analysis) and c.7589G>A (using TaqI restriction analysis) (Mendive *et al.* 1997, Gutnisky *et al.* 2004), Tgms1, Tgms2, TGrI29, and TGrI30 microsatellites and the IndelIVSTG18 markers were used to determine the allelic distribution in the family LD. Specific haplotypes were identified for the p.R277X and p.R1511X mutated alleles and normal parental alleles. As shown in Figs 2 and 3*a*, in LD's father (LA), the 307 bp Tgms1, 541 bp IndelIVSTG18, cytosine 4506, 340 bp Tgms2, 201 bp TGrI29, 538 bp TGrI30, and guanine 7589 alleles are associated with the presence of the p.R277X mutation, whereas in LD's mother (LG) the 307 bp Tgms1, 541 bp IndelIVSTG18, cytosine 4506, 338 bp Tgms2, 201 bp TGrI29, 538 bp TGrI30, and guanine 7589 alleles are associated with the presence of the p.R1511X mutation.

Comparative analysis of the polymorphic markers in the TG gene

In order to discriminate between a *de novo* recurrence of the p.R277X and p.R1511X mutations and a founder effect, we compared the haplotypes identified in the index LD patient with the haplotypes from the II-2 member of the MA family. The fifteen exonic TG SNPs, IndelIVSTG18, Tgms1, Tgms2, TGrI29, and TGrI30 markers were used for haplotype analysis. The presence of exonic SNPs was evaluated by sequence analysis. The allele designation to p.R277X or p.R1511X mutations in each marker that was found heterozygous were inferred with the aid of father and mother polymorphism data for LD or father polymorphism data for II-2. Mother of II-2 was not available. The father analysis was not informative for allele designation of the 7501T>C and 7920C>T SNPs in II-2, which had previously been identified from other member of the family, III-2 (Rivolta *et al.* 2005).

The IndelIVSTG18, microsatellites TGrI29 and TGrI30, and SNPs 229G>A, c.2488C>G, c.3474T>C, c.3935G>A, c.4506C>T, c.5512A>G, c.6695C>T, c.7408C>T, and c.7589G>A results showed that the two affected individuals are homozygous for the same allele in each

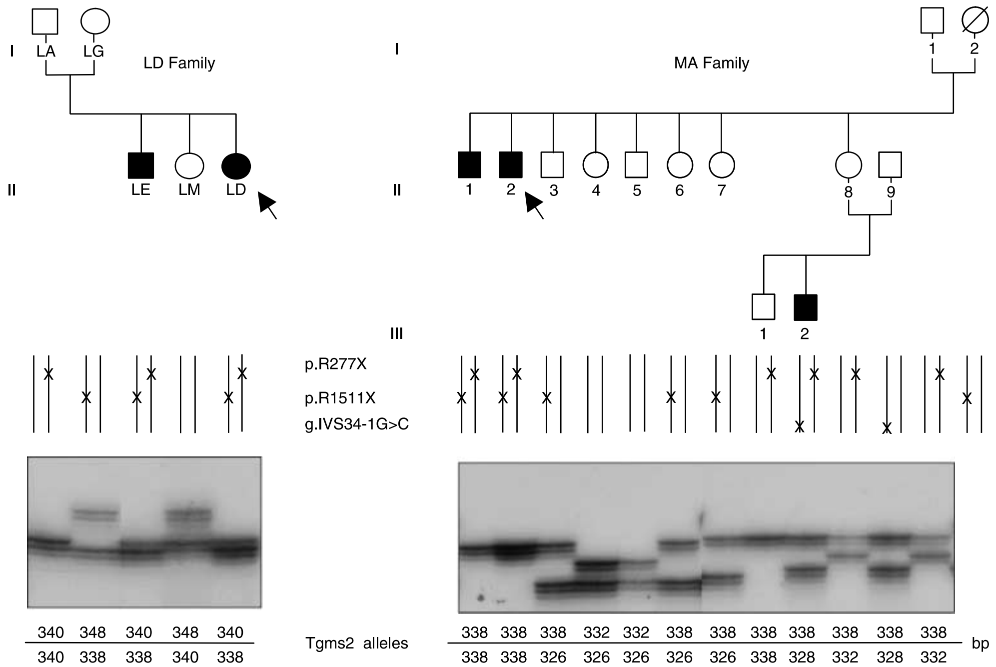

Figure 2 Autoradiograph showing alleles at Tgms2 locus for members of the LD and MA families. The pedigree shows the pattern of inheritance of the mutant thyroglobulin alleles. All data are aligned with each individual's symbol on the pedigree. Square and circle symbols indicate male and female members respectively. Filled symbols denote affected individuals by congenital goiter and hypothyroidism. The solid arrow indicates the index LD patient and II-2. Molecular weights are indicated in bps (bp).

marker, consequently were not informative polymorphisms for this study (Fig. 3*b*). However, the Tgm1 and the Tgm2 microsatellites and c.2200T>G, c.2334T>C, c.3082A>G, c.7501T>C, and c.7920C>T SNPs analysis revealed that LD and II-2 do not share TG alleles associated with the p.R277X mutation (Fig. 3*b*). LD carries 307 bp Tgms1 and 340 bp Tgms2 alleles, whereas II-2 carries 305 bp Tgms1 and 338 bp Tgms2 alleles. LD harbors G, C, G, T, and C in the SNPs localized in the nucleotide positions 2200, 2334, 3082, 7501, and 7920T respectively. In contrast, II-2 harbors T, T, A, C, and T in the same SNPs. This strongly suggested that the p.R277X alleles are due to independently recurrent mutations.

The c.5995C>T, c.7501T>C, and c.7920C>T SNPs analyses showed also that LD and II-2 do not share TG alleles associated with the p.R1511X mutation (Fig. 3*b*). LD has C, C, and T for this SNPs, whereas II-2 has T, T, and C for the same SNPs. According with these results, it is very likely that the p.R1511X mutation is also an independent mutational event.

Finally, comparative analysis between the haplotypes segregation with the mutation p.R277X from the LD family and a previously reported Argentinian RM patient with the same mutation in homozygous state (Rivolta *et al.* 2005) showed the same combinations of polymorphisms for one p.R277X allele (Fig. 3*b*) and the remaining allele possessed a single SNP different in the heterozygous state (p.R1980W).

Population screening for the p.R277X and p.R1511X mutations

To investigate the possibility that the p.R277X and p.R1511X mutations had a significant frequency in the general population, a mutational screening was performed in 250 unrealed healthy subjects as well as in 40 patients with sporadic nonendemic simple goiter. The presence of the 886C>T and 4588C>T mutations were analyzed by differential restriction analysis with AlwN I and TaqI respectively (Fig. 4). Both nonsense mutations were not detected in the 580 chromosomes investigated. Consequently, our study confirms the very low prevalence of the TG mutations in the general population.

Discussion

We report two siblings of Argentinian origin with congenital hypothyroidism and goiter due to TG deficiency. The diagnosis of dyshormonogenesis was based on the lower serum TG, elevated serum TSH with simultaneous low serum T_4 levels and perchlorate discharge test interpreted as negative (Rivolta & Targovnik 2006). Molecular analyses revealed a p.R277X/p.R1511X compound heterozygous mutation, which has been found before causing TG deficiency (Targovnik *et al.* 1993, van de Graaf *et al.* 1999, Gutnisky *et al.* 2004, Mendive *et al.* 2005, Rivolta *et al.* 2005).

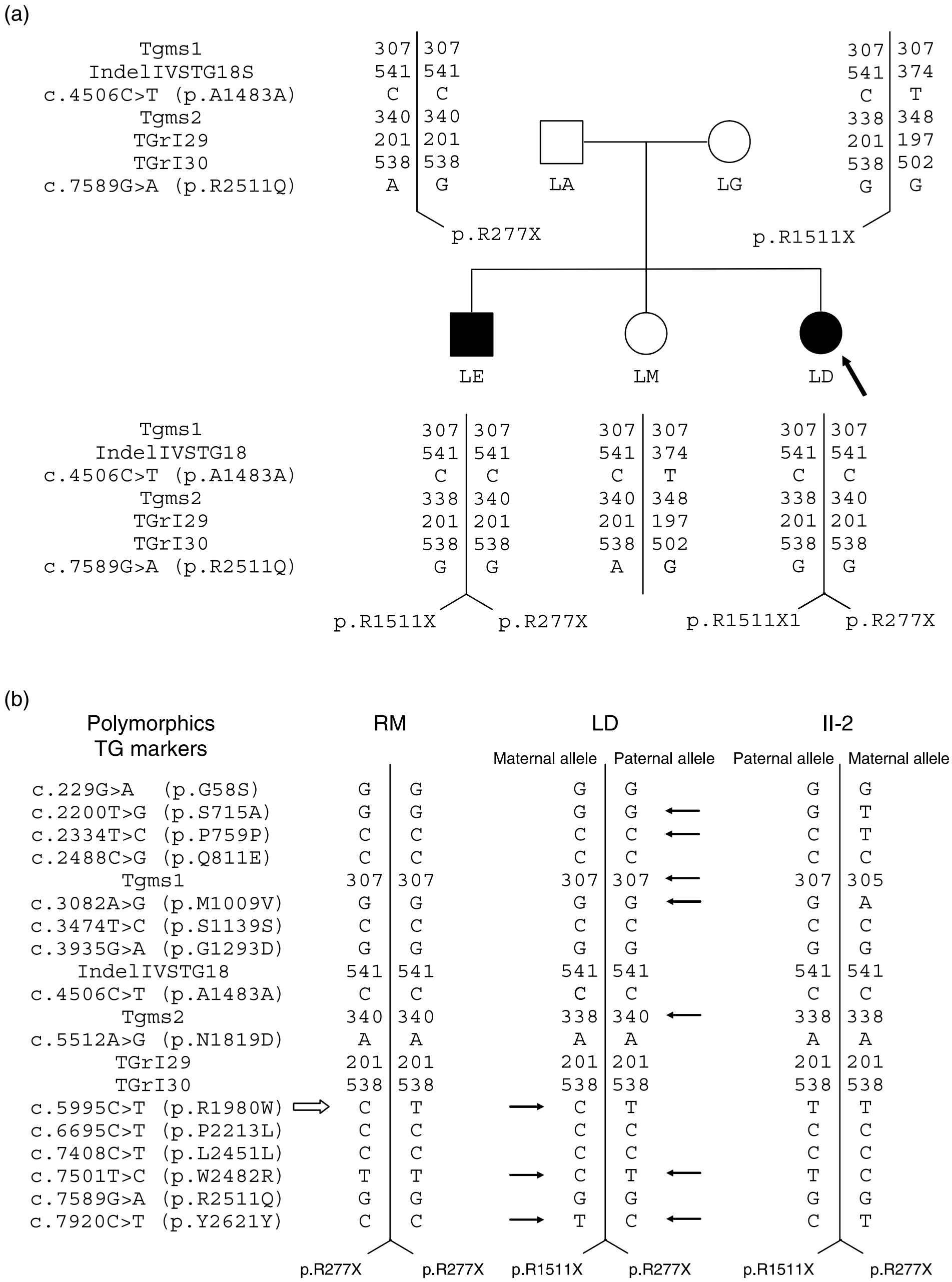

Figure 3 Haplotype analyses. (a) Pedigree of the LD family showing haplotype analyses using seven polymorphic markers. The pedigree shows the pattern of inheritance of the mutant thyroglobulin alleles. All data are aligned with each individual's symbol on the pedigree. Note that both affected siblings LD and LE have inherited one copy of the p.R277X mutation from their father (LF) and one copy of the p.R1511X mutation from their mother (LA). Square and circle symbols indicate male and female members respectively. Filled symbols denote affected individuals by congenital goiter and hypothyroidism. The solid arrow indicates the index patient LD. (b) Comparative haplotype analysis of the RM, LD, and II-2 patients using 15 SNPs, 4 microsatellites, and 1 insertion/deletion polymorphism markers. The solid arrows indicate the informative polymorphic markers for the association to p.R1551X (left) or p.R277X (right) mutated alleles between LD and II-2. The open arrow denotes a single SNP different in RM. The parents of RM were not available for segregation analysis.

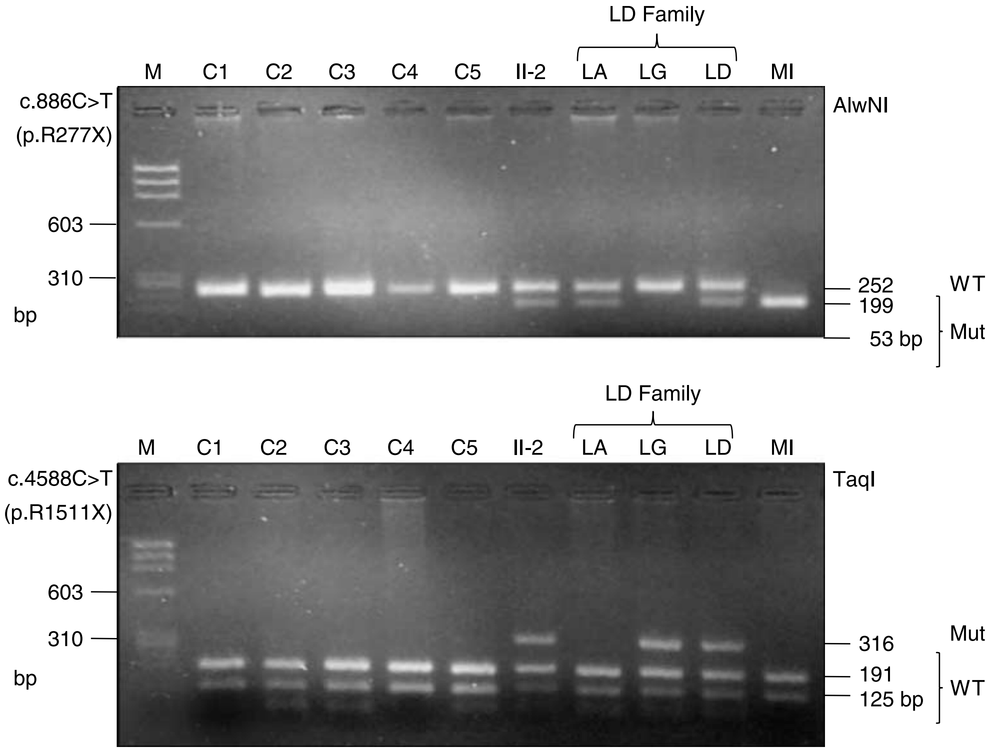

Figure 4 Population screening for the p.R277X and p.R1511X mutations. The exons 7 and 22 PCR fragments were digested with AlwNI and TaqI respectively, then subjected to electrophoresis in a 2·5% agarose gel. After staining with ethidium bromide, the gel was photographed under u.v. light. The wild-type (WT) and mutate (Mut) restriction bands are indicated. Molecular weights are indicated in bps (bp). ϕX174 RF DNA/HaeIII fragments were used as a DNA size marker in lane M. AlwNI restriction analysis showed that the affected II-2 patient, LD's father (LA) and the index LD patient exhibit a mutant and wild-type alleles for p.R277X. The LD's mother (LG) and unrealed healthy subjects (C1, C2, C3, C4, C5) have only the wild-type alleles at this position and MI, control p.R277X homozygote, has only mutant alleles. TaqI restriction analysis showed that the affected II-2 patient, LD's mother (LG) and the index LD patient exhibit a mutant and wild-type alleles for p.R1511X. The LD's father (LA), MI, and unrealed healthy subjects (C1, C2, C3, C4, C5) have only the wild-type alleles at this position.

The monomer of TG is composed of a 19 amino acid signal peptide followed by 2749 residues containing 66 tyrosines (Malthièry & Lissitzky 1987, Rivolta & Targovnik 2006). The monomeric primary structure is characterized by the presence of three types of repetitive units that include 11 type-1, 3 type-2, and 5 type-3 repeat motifs (Malthièry & Lissitzky 1987, van de Graaf *et al.* 1997, 2001, Mendive *et al.* 2001, Rivolta & Targovnik 2006). However, the carboxy-terminal domain of the molecule is not repetitive and shows a striking homology with acetylcholinesterase (ACHE-like domain; Swillens *et al.* 1986, Park & Arvan 2004). This suggests a probable convergent origin of the TG gene from different ancestral DNA sequences. A correct three-dimensional structure is essential for thyroid hormonogenesis. Once TG has reached the follicular lumen, several tyrosine residues are iodinated and certain iodinated tyrosines are coupled to form T_3 and T_4. Four hormonogenic acceptor tyrosines have been identified and localized at positions 5, 1291, 2554, and 2747 in human TG and several tyrosines localized at positions 130, 847, and 1448 have been proposed as outer ring donor sites (Malthièry & Lissitzky 1987, van de Graaf *et al.* 1997, 2001, Mendive *et al.* 2001, Rivolta & Targovnik 2006). The most important hormonogenic acceptor site is at tyrosine 5 which couples with the donor tyrosine at position 130 (Dunn *et al.* 1998).

The p.R277X mutation results in a grossly truncated protein of 276 amino acids with limited ability to generate thyroid hormone. However, the truncated form of TG still harbors the acceptor tyrosine 5 and the donor tyrosine 130 and eliminates the carboxy-terminal hormonogenic domain. Previous study excluded an alternative splicing mechanism, by skipping exon 7, in order to restore the normal reading frame disrupted by the p.R277X and eliminate the stop codon which would truncate the protein (van de Graaf *et al.* 1999, Rivolta *et al.* 2005). In contrast, the p.R1511X mutation is removed from the transcripts by exon skipping and there is a preferential accumulation in the goiter of a TG mRNA lacking exon 22 (Targovnik *et al.* 1993). The deletion does not affect the reading frame of the resulting mRNA and generates a TG polypeptide chain that is shortened by 57 residues.

Interestingly, the maternal LD haplotype and paternal MA haplotype are identical in the 5′ region of the gene down to c.5995C>T SNP. Major deletions involving this region can be disregarded because of the presence in the exon 7 of the p.R277X mutation in the heterozygous state (Fig. 1*a*).

It is of clinical and public health interest to know whether a mutation is an independently recurrent mutation or whether it is due to a founder effect. In this sense, we genotyped a set of 4 microsatellites, 1 indel, and 15 exonic SNPs localized in the TG gene from the index LD patient and a affected individual, II-2, from a previously reported unrelated Brazilian family with congenital goiter and hypothyroidism harboring also the p.R277X/p.R1551X compound heterozygous mutation (Targovnik *et al*. 1993, Gutnisky *et al*. 2004, Mendive *et al*. 2005). LD has inherited one copy of the p.R277X mutation from their father, and one copy of the p.R1511X mutation from their mother, whereas II-2 has inherited one copy of the p.R277X mutation from their mother, and one copy of the p.R1511X mutation from their father. Different haplotypes segregated with the p.R277X and p.R1511X mutations in each patient demonstrating the absence of a founder effect for these mutations between Argentinian and Brazilian families (Fig. 3*b*). The nonsense mutation in exons 7 and 22 occurs in a CpG dinucleotide sequence and could be caused by deamination of a methylated cytosine resulting in a thymine (Krawczak *et al*. 1998). The CGA arginine codon is considered a hot spot for mutations in mammalian DNA. However, comparative analysis between the haplotypes segregation with the mutation p.R277X from the LD family and a previously described Argentinian RM patient with congenital hypothyroidism due to the same mutation in homozygous state (Rivolta *et al*. 2005) showed the same combinations of intragenic TG polymorphisms except for the p.R1980W (Fig. 3*b*). Consequently, this is a strong indication that the p.R277X alleles in Argentinian families might be derived from a common ancestral chromosome. It is also likely that p.R277X is an old mutation and p.R1980W is a new SNP.

In conclusion, we report a new case with congenital goitrous hypothyroidism caused by the p.R277X/p.R1511X compound heterozygous mutation in the TG gene. Analysis of mutation-associated haplotypes by genotyping all family members suggests the absence of a founder effect for the p.R1511X and p.R277X mutations between Argentinian and Brazilian populations. We therefore suggest a possible common origin for the Argentinian p.R277X alleles. The identification of a new case of congenital hypothyroidism due to p.R277X mutations in the TG gene helps to expand our knowledge on the mutational mechanism responsible for this mutation. In addition, the results of the present investigation show clearly the very low prevalence of the TG mutations in the general population.

Acknowledgements

M C is a research fellow of the Universidad de Buenos Aires. C M R is an established investigator of the Argentine National Research Council (CONICET) and a visiting investigator at the Unidad de Medicina Molecular, Universidad de Salamanca, from September 2005, to January 2006, supported by grants from CONICET and Coimbra Group. H M T is an established investigator of the CONICET and a visiting professor at the Unidad de Medicina Molecular, Universidad de Salamanca, from November 2003, to May 2004 and from September 2005, to February 2006. This work was supported by grants from Universidad de Buenos Aires (B 057/2004 to HMT), CONICET (5360/2005 to CMR and HMT), ANPCyT-FONCyT (05-21081/PICT 2004 to HMT), and Consejería de Cultura y Bienestar Social, Castilla y León (SA112A05 to RG-S). The authors are grateful to Nieves Mateos and Manuel Sánchez for their excellent technical assistance. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References

Alzahrani AS, Baitei EY, Zou M & Shi Y 2006 Metastatic thyroid follicular carcinoma arising from congenital goiter due to a novel splice donor site mutation in the thyroglobulin gene. *Journal of Clinical Endocrinology and Metabolism* **91** 740–746.

Caron P, Moya CM, Malet D, Gutnisky VJ, Chabardes B, Rivolta CM & Targovnik HM 2003 Compound heterozygous mutations in the thyroglobulin gene (1143delC and 6725GA(R2223H)) resulting in fetal goitrous hypothyroidism. *Journal of Clinical Endocrinology and Metabolism* **88** 3546–3553.

Corral J, Martín C, Pérez R, Sánchez I, Mories MT, San Millan JL, Miralles JM & González-Sarmiento R 1993 Thyroglobulin gene point mutation associated with non-endemic simple goitre. *Lancet* **341** 462–464.

Dunn AD, Corsi CM, Myers HE & Dunn JT 1998 Tyrosine 130 is an important outer ring donor for thyroxine formation in thyroglobulin. *Journal of Biological Chemistry* **273** 25223–25229.

González-Sarmiento R, Corral J, Mories MT, Corrales JJ, Miguel-Velado E & Miralles-Garcia JM 2001 Monoallelic deletion in the 5′ region of the thyroglobulin gene as a cause of sporadic nonendemic simple goiter. *Thyroid* **11** 789–793.

Gutnisky VJ, Moya CM, Rivolta CM, Domené S, Varela V, Toniolo JV, Medeiros-Neto G & Targovnik HM 2004 Two distinct compound heterozygous constellation (R277X/IVS34-1G>C and R277X/R1511X) in the thyroglobulin (TG) gene in affected individuals of a brazilian kindred with congenital goiter and defective TG synthesis. *Journal of Clinical Endocrinology and Metabolism* **89** 646–657.

Hishinuma A, Takamatsu J, Ohyama Y, Yokozawa T, Kanno Y, Kuma K, Yoshida S, Matsuura N & Ieiri T 1999 Two novel cysteine substitutions (C1263R and C1995S) of thyroglobulin cause a defect in intracellular transport of thyroglobulin in patients with congenital goiter and the variant type of adenomatous goiter. *Journal of Clinical Endocrinology and Metabolism* **84** 1438–1444.

Hishinuma A, Furudate S-I, Masamichi O-I, Nagakubo N, Namatame T & Ieiri T 2000 A novel missense mutation (G2320R) in thyroglobulin causes hypothyroidism in rdw rats. *Endocrinology* **141** 4050–4055.

Hishinuma A, Fukata S, Kakudo K, Murata Y & Ieiri T 2005 High Incidence of thyroid cancer in long-standing goiters with thyroglobulin mutations. *Thyroid* **15** 1079–1084.

Hishinuma A, Fukata S, Nishiyama S, Nishi Y, Oh-Ishi M, Murata Y, Ohyama Y, Matsuura N, Kasai K, Harada S *et al*. 2006 Haplotype analysis reveals founder effects of thyroglobulin gene mutations C1058R and C1977S in Japan. *Journal of Clinical Endocrinology and Metabolism* **91** 3100–31004.

Ieiri T, Cochaux P, Targovnik HM, Suzuki M, Shimoda S-I, Perret J & Vassart G 1991 A 3′ splice site mutation in the thyroglobulin gene responsible for congenital goiter with hypothyroidism. *Journal of Clinical Investigation* **88** 1901–1905.
Kim PS, Hossain SA, Park Y-N, Lee I, Yoo S-E & Arvan P 1998 A single amino acid change in the acetylcholinesterase-like domain of thyroglobulin causes congenital goiter with hypothyroidism in the cog/cog mouse: a model of human endoplasmic reticulum storage diseases. *PNAS* **95** 9909–9913.
Kim PS, Ding M, Menon S, Jing C-G, Cheng J-M, Miyamoto T, Li B, Furudate S-I & Agui T 2000 A missemse mutation G2320R in the thyroglobulin gene causes non-goitrous congenital primary hypothyroidism in the WIC-rdw rat. *Molecular Endocrinology* **14** 1944–1953.
Kitanaka S, Takeda A, Sato U, Miki Y, Hishinuma A, Ieiri T & Igarashi T 2006 A novel compound compound heterozygous mutation in the thyroglobulin gene resulting in congenital goitrous hypothyroidism with high serum triiodothyronine levels. *Journal of Human Genetics* **51** 379–382.
Krawczak M, Ball EV & Cooper DN 1998 Neighboring-nucleotide effects on the rates of germ-line single-base-pair substitution in human genes. *American Journal of Human Genetics* **63** 474–488.
Malthièry Y & Lissitzky S 1987 Primary structure of human thyroglobulin deduced from the sequence of its 8448-base complementary DNA. *European Journal of Biochemistry* **165** 491–498.
Medeiros-Neto G, Kim PS, Yoo SE, Vono J, Targovnik HM, Camargo R, Hossain SA & Arvan P 1996 Congenital hypothyroid goiter with deficient thyroglobulin. Identification of an endoplasmic reticulum storage disease with induction of molecular chaperones. *Journal of Clinical Investigation* **98** 2838–2844.
Mendive FM, Rossetti LC, Vassart G & Targovnik HM 1997 Identification of a new thyroglobulin variant: a guanine-to-arginine transition resulting in the substitution of arginine 2510 by glutamine. *Thyroid* **7** 587–591.
Mendive FM, Rivolta CM, Moya CM, Vassart G & Targovnik HM 2001 Genomic organization of the human thyroglobulin gene. The complete intron–exon structure. *European Journal of Endocrinology* **145** 485–496.
Mendive FM, Rivolta CM, González-Sarmiento R, Medeiros-Neto G & Targovnik HM 2005 Nonsense-associated alternative splicing of the human thyroglobulin gene. *Molecular Diagnosis* **9** 143–149.
Moya CM, Varela V, Rivolta CM, Mendive FM & Targovnik HM 2003 Identification and characterization of a novel large insertion/deletion polymorphism of 1464 base pair in the human thyroglobulin gene. *Thyroid* **13** 319–323.
Murray MG & Thompson WF 1980 Rapid isolation of high molecular-weight plant DNA. *Nucleic Acids Research* **8** 4321–4325.
Park YN & Arvan P 2004 The acetylcholinesterase homology region is essential for normal conformational maturation and secretion of thyroglobulin. *Journal of Biological Chemistry* **279** 17085–17089.
Pérez-Centeno C, González-Sarmiento R, Mories MT, Corrales JJ & Miralles-García JM 1996 Thyroglobulin exon 10 gene point mutation in a patient with endemic goiter. *Thyroid* **6** 423–427.
Ricketts MH, Simons MJ, Parma J, Mercken L, Dong Q & Vassart G 1987 A nonsense mutation causes hereditary goitre in the Afrikander cattle and unmasks alternative splicing of thyroglobulin transcripts. *PNAS* **84** 3181–3184.
Rivolta CM & Targovnik HM 2006 Molecular advances in thyroglobulin disorders. *Clinica Chimica Acta* **374** 8–24.
Rivolta CM, Moya CM, Mendive FM & Targovnik HM 2002 Genotyping and characterization of two polymorphic microsatellite markers located within introns 29 and 30 of the human thyroglobulin gene. *Thyroid* **12** 773–779.
Rivolta CM, Moya CM, Gutnisky VJ, Varela V, Miralles-García JM, González-Sarmiento R & Targovnik HM 2005 A new case of congenital goiter with hypothyroidism due to a homozygous p.R277X mutation in the exon 7 of the thyroglobulin gene: a mutational hot spot could explain the recurrence of this mutation. *Journal of Clinical Endocrinology and Metabolism* **90** 3766–3770.
Swillens S, Ludgate M, Mercken L, Dumont JE & Vassart G 1986 Analysis and structure homologies between thyroglobulin and acetylcholinesterase: possible functional and clinical significance. *Biochemical and Biophysical Research Communications* **137** 142–148.
Targovnik HM, Varela V, Juvenal GJ, Propato F, Chester HA, Krawiec L, Frechtel G, Moran DH, Perinetti HA & Pisarev MA 1990 Differential levels of thyroid peroxidase and thyroglobulin messenger ribonucleic acids in congenital goiter with defective thyroglobulin synthesis. *Journal of Endocrinological Investigation* **13** 797–806.
Targovnik HM, Medeiros-Neto G, Varela V, Cochaux P, Wajchenberg BL & Vassart G 1993 A nonsense mutation causes human hereditary congenital goiter with preferential production of a 171-nucleotide-deleted thyroglobulin ribonucleic acid messenger. *Journal of Clinical Endocrinology and Metabolism* **77** 210–215.
Targovnik H, Vono J, Billerbeck AEC, Cerrone GE, Varela V, Mendive F, Wajchenberg BL & Medeiros-Neto G 1995 A 138-nucleotide deletion in the thyroglobulin ribonucleic acid messenger in a congenital goiter with defective thyroglobulin synthesis. *Journal of Clinical Endocrinology and Metabolism* **80** 3356–3336.
Targovnik HM, Rivolta CM, Mendive FM, Moya CM & Medeiros-Neto G 2001 Congenital goiter with hypothyroidism caused by a 5′ splice site mutation in the thyroglobulin gene. *Thyroid* **11** 685–690.
Tomer Y, Greenberg DA, Concepcion E, Ban Y & Davies TF 2002 Thyroglobulin is a thyroid specific gene for the familial autoimmune thyroid diseases. *Journal of Clinical Endocrinology and Metabolism* **87** 404–407.
van de Graaf SAR, Pauws E, de Vjilder JJM & Ris-Stalpers C 1997 The revised 8307 base pair coding sequence of human thyroglobulin transiently expressed in eukaryotic cells. *European Journal of Endocrinology* **136** 508–515.
van de Graaf SAR, Ris-Stalpers C, Veenboer GJM, Cammenga M, Santos C, Targovnik HM, de Vijlder JJM & Medeiros-Neto G 1999 A premature stopcodon in thyroglobulin mRNA results in familial goiter and moderate hypothyroidism. *Journal of Clinical Endocrinology and Metabolism* **84** 2537–2542.
van de Graaf SAR, Ris-Stalpers C, Pauws E, Mendive FM, Targovnik HM & de Vijlder JJM 2001 Up to date with human thyroglobulin. *Journal of Endocrinology* **170** 307–321.
Veenboer GJM & de Vijlder JJM 1993 Molecular basis of the thyroglobulin synthesis defect in Dutch goats. *Endocrinology* **132** 377–381.

Received in final form 25 July 2007
Accepted 8 August 2007
Made available online as an Accepted Preprint
9 August 2007