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New insights into thyroglobulin gene: Molecular analysis of seven novel mutations associated with goiter and hypothyroidism

Cintia E. Citterio [a,b], Gloria A. Machiavelli [a,b], Mirta B. Miras [c], Laura Gruñeiro-Papendieck [d], Katherine Lachlan [e], Gabriela Sobrero [c], Ana Chiesa [d], Joanna Walker [f], Liliana Muñoz [c], Graciela Testa [c], Fiorella S. Belforte [a,b], Rogelio González-Sarmiento [g], Carina M. Rivolta [a,b], Héctor M. Targovnik [a,b,*]

[a] Laboratorio de Genética y Biología Molecular, Instituto de Inmunología, Genética y Metabolismo (INIGEM, CONICET-UBA), Hospital de Clínicas "José de San Martín", C1120AAR Buenos Aires, Argentina
[b] Cátedra de Genética y Biología Molecular (FFyB-UBA), C1113AAD Buenos Aires, Argentina
[c] Servicio de Endocrinología, Hospital de Niños Santísima Trinidad, 5000 Córdoba, Argentina
[d] Centro de Investigaciones Endocrinológicas, CEDIE-CONICET, División Endocrinología, Hospital de Niños "Ricardo Gutiérrez", C1425EFD Buenos Aires, Argentina
[e] Wessex Clinical Genetics Service, University Hospital Southampton, Princess Anne Hospital, SO16 5YA Southampton, United Kingdom
[f] Portsmouth Hospitals NHS Trust, Queen Alexandra Hospital, Cosham, PO6 3LY Portsmouth, United Kingdom
[g] Unidad de Medicina Molecular-Departamento de Medicina, IBMCC and IBSAL, Universidad de Salamanca-CSIC, 37007 Salamanca, Spain

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ABSTRACT

The thyroglobulin (TG) gene is organized in 48 exons, spanning over 270 kb on human chromosome 8q24. Up to now, 62 inactivating mutations in the TG gene have been identified in patients with congenital goiter and endemic or non-endemic simple goiter.

The purpose of the present study was to identify and characterize new mutations in the TG gene. We report 13 patients from seven unrelated families with goiter, hypothyroidism and low levels of serum TG. All patients underwent clinical, biochemical and imaging evaluation. Single-strand conformation polymorphism (SSCP) analysis, endonuclease restriction analysis, sequencing of DNA, genotyping, population screening, and bioinformatics studies were performed.

Molecular analyses revealed seven novel inactivating TG mutations: c.378C>A [p.Y107X], c.2359C>T [p.R768X], c.2736delG [p.R893fsX946], c.3842G>A [p.C1262Y], c.5466delA [p.K1803fsX1833], c.6000C>G [p.C1981W] and c.6605C>G [p.P2183R] and three previously reported mutations: c.886C>T [p.R277X], c.6701C>A [p.A2215D] and c.7006C>T [p.R2317X]. Six patients from two families were homozygous for p.R277X mutation, four were compound heterozygous mutations (p.Y107X/p.C1262Y, p.R893fsX946/p.A2215D, p.K1803fsX1832/p.R2317X), one carried three identified mutations (p.R277X/p.C1981W-p.P2183R) together with a hypothetical micro deletion and the remaining two siblings from another family with typical phenotype had a single p.R768X mutated allele.

In conclusion, our results confirm the genetic heterogeneity of TG defects and the pathophysiological importance of altered TG folding as a consequency of truncated TG proteins and missense mutations located in ACHE-like domain or that replace cysteine.

1. Introduction

Thyroid dyshormonogenesis due to thyroglobulin (TG) gene mutations have an estimated incidence of approximately 1 in 100,000 newborns (Targovnik et al., 2010a, 2011). The clinical spectrum ranges from euthyroid to mild or severe hypothyroidism. The majority of patients have congenital goiter or goiter appearing shortly after birth (Targovnik et al., 2010a, 2011).

Human TG gene is a single copy gene, 270 kb long which maps on chromosome 8q24 and contains an 8.5-kb coding sequence divided into 48 exons (Targovnik et al., 2010a, 2011). Transcription of TG is highly specific to the thyroid cells and is under control of the coordinated action of a master set of transcription factors that includes the homeodomain protein NKX2.1 (TTF-1), the forkhead-domain protein FOXE1 (TTF-2) and the paired-domain protein PAX8 (Targovnik et al., 2010a, 2011). However, a recently report showed that some chondrocytes have the ability to express TG (Endo and Kobayashi, 2011).

TG is a large homodimeric secretory protein with a high degree of glycosylation. The preprotein monomer is composed of a leader peptide of 19 amino acids followed by 2748-amino-acid

* Corresponding author. Address: Laboratorio de Genética y Biología Molecular, Instituto de Inmunología, Genética y Metabolismo (INIGEM, CONICET-UBA), Hospital de Clínicas "José de San Martín", Av. Córdoba 2351, Cuarto Piso, Sala 5, C1120AAR Buenos Aires, Argentina. Tel.: +54 11 5950 8805.
E-mail address: htargovnik@conicet.gov.ar (H.M. Targovnik).

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polypeptide (van de Graaf et al., 2001; Targovnik, 2012). Its main function is to provide the macromolecular precursor for synthesis and storage of thyroid hormones, thyroxine (T_4) and triiodothyronine (T_3). It is also an important storage of iodine when external odine availability is limited (Targovnik, 2012). Eighty percent of the overall TG monomer encloses three regions with repeat domains (van de Graaf et al., 2001; Lee et al., 2011; Targovnik, 2012). 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 remaining 20%, that constitutes the carboxy-terminal domain of the molecule, is not repetitive and shows significant homology with the acetylcholinesterase (ACHE-like domain) (van de Graaf et al., 2001; Lee et al., 2011; Targovnik, 2012).

Sixty-two mutations have been identified and characterized in the human TG: 14 splice site mutations, 12 nonsense mutations, 25 missense mutations, eight deletions (six single and two involving a large number of nucleotides) and three single nucleotide insertions (Ieiri et al., 1991; Corral et al., 1993; Targovnik et al., 1993, 1995, 2001, 2010b, 2012; Pérez-Centeno et al., 1996; Hishinuma et al., 1999, 2005, 2006; van de Graaf et al., 1999; González-Sarmiento et al., 2001; Caron et al., 2003; Gutnisky et al., 2004; Rivolta et al., 2005; Alzahrani et al., 2006; Kitanaka et al., 2006; Caputo et al., 2007a,b; Kanou et al., 2007; Kim et al., 2008; Pardo et al., 2008, 2009; Niu et al., 2009; Machiavelli et al., 2010; Peteiro-Gonzalez et al., 2010; Raef et al., 2010; Citterio et al., 2011; Narumi et al., 2011; Moya et al., 2011; Kahara et al., 2012; Liu et al., 2012). Because TG mutations are inherited in an autosomal recessive manner, the patients should be homozygous or compound heterozygous for gene mutations and the parents should be carriers of one TG mutation.

In the present study we report 13 patients from seven unrelated families with goiter, hypothyroidism, and low levels of serum TG. Analysis of the TG gene revealed seven novel mutations: p.Y107X, p.R768X, p.R893fsX946, p.C1262Y, p.K1803fsX1832, p.C1981W, and p.P2183R, and three previously reported mutations: p.R277X (van de Graaf et al., 1999; Gutnisky et al., 2004; Rivolta et al., 2005; Caputo et al., 2007a,b; Pardo et al., 2009; Machiavelli et al., 2010; Peteiro-Gonzalez et al., 2010; Citterio et al., 2011), p.A2215D (Caputo et al., 2007a; Pardo et al., 2008, 2009; Machiavelli et al., 2010), and p.R2317X (Machiavelli et al., 2010; Liu et al., 2012). Six patients from two families were homozygous for p.R277X mutation, four were compound heterozygous mutations (p.Y107X/p.C1262Y, p.R893fsX946/p.A2215D, p.K1803fsX1832/p.R2317X), one carried three identified mutations (p.R277X/p.C1981W-p.P2183R) together with a hypothetical micro deletion and the remaining two siblings from another family with typical phenotype had a single p.R768X mutated allele.

2. Materials and methods

2.1. Patients

Patients selected to participate in this study had goiter, hypothyroidism, elevated serum TSH, low serum total T_4 levels with simultaneous low or normal serum T_3 levels, low serum TG concentration and negative anti-TG and anti-TPO antibodies. Laboratory testing is shown in Table 1 and Fig. 1. All the patients came from iodide-sufficient areas. Families A (III-1, III-2 and III-3), B (II-1, II-2 and II-3), C (II-4), D (II-3 and II-4) and E (II-1) were followed at Endocrine Unit of 'Hospital de Niños Santísima Trinidad', family F (II-2) was followed at Endocrine Division of 'Hospital de Niños Ricardo Gutiérrez', and family G (II-1 and II-2) was followed at Endocrinology Department of Queen Alexandra Hospital. The family pedigrees are shown in Fig. 1.

Therapy with L-T_4 was initiated at doses shown in Table 1 and adjusted according to weight and TSH levels during the years following at diagnosis. In all patients the goiter remained after long-term substitution therapy. However, 6 of them detected by newborn screening (II-4 of family C, II-3 and II-4 of family D, II-1 of family E, and II-1 of family G) or fetal ultrasound (II-2 of family G) did not show development of large goiters, confirming the beneficial effects of early treatment.

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

2.1.1. Family A

2.1.1.1. Patient III-1. The patient is the first child of an unrelated couple. He was referred to the endocrinnology centre at the age of 15.8 due to short stature, goiter, clinical signs of hypothyroidism and mental retardation. At the age of 7, he was diagnosed with hypothyroidism in another institution and received replacement therapy in an interrupted manner. He was not evaluated through the neonatal screening program because the test was not still widely implemented at the time of his birth. At present, he attends a special school.

2.1.1.2. Patient III-2. This patient is III-1's brother diagnosed at 18 days of life, through the neonatal screening program. Since then, he was treated with L-T_4 replacement therapy irregularly for 3 years; parents decided to stop therapy between 3 and 5 years of age. He was referred to the endocrinology center at the age of 5.9 due to short stature, swollen facies, pale skin, goiter and mental retardation. He attends primary school and has learning difficulties, he receives psychopedagogical assistance.

2.1.1.3. Patient III-3. He is patient III-1's younger brother. His congenital hypothyroidism was detected at 42 days of life. He was born at term after a noncomplicated pregnancy and delivery. Clinical examination showed goiter, jaundice and clamping of the umbilical cord at 13 days. He grew up without developmental disturbance or intellectual impairment.

2.1.2. Family B

2.1.2.1. Patient II-1. She is the first child of a non-consanguineous couple referred from the rural area at 16 years of age due to short stature, goiter and mental retardation. Ultrasound showed an enlargement of the gland (Table 1) with heterogeneous pattern of micronodular appearance. In the lower region of left thyroid lobe there is a nodule image of 28×9 mm.

2.1.2.2. Patient II-2. This patient is II-1's sister diagnosed with congenital hypothyroidism at the age of 14. At presentation, she showed short stature, convergent strabismus, swollen facies, dry and cold skin, swollen abdomen and a very large asymmetrical goiter, with predominance of the left lobe and of soft consistency. Thyroid ultrasound showed a big enlargement of the gland (Table 1) with a heterogeneous pattern. She had severe psycho-intellectual retardation and did not finish primary school.

2.1.2.3. Patient II-3. Patient II-3, patient II-1's sister, was referred at 12 years of age due to short stature, signs and symptoms of hypothyroidism and a very large asymmetrical goiter, with increasing the consistency. Thyroid ultrasound showed an enlarged gland (Table 1) with inhomogeneous pattern. Right thyroid lobe was dominated by a solid macronodule. Left thyroid lobe showed macro and micronodular patterns all along, the dominant one being of 4 mm. No adenopathies were observed. Fine-needle aspiration biopsy showed a hyperplastic nodular goiter associated with thyroiditis.

Table 1
Clinical and laboratory data of patients with goiter, hypothyroidism and thyroglobulin defect.

Familias	Patients	Gender	Age at diagnosis	Ultrasound Thyroid size ml	^{99}Tc Scan Thyroid size	Serum TSH (mIU/L)	Serum TT_4 (μg/dl)	Serum FT_4 (ng/dl)	Serum TT_3 (ng/dl)	Serum TG (ng/ml)	L-T_4 replacement therapy (μg/kg/day)
A	III-1	M	7 yrs/ re-evaluation at 15.8 yrs	ND	ND	150	0.25	0.12	60	1	2.8
	III-2	M	18 ds/ re-evaluation at 5.9 yrs	ND	Enlarged HDR	150	0.25	0.12	53	1	5.6
	III-3	M	42 ds	3.6	Enlarged HDR	725	1.8	0.8	100	1	8.0
B	II-1	F	16 yrs	42	ND	58	ND	0.38	ND	ND	2.2
	II-2	F	14 yrs	271	ND	52	1.25	0.19	99	1	2.5
	II-3	F	12 yrs	151	ND	100	0.56	0.09	68.3	1	2.5
C	II-4	F	9 ds	7	Enlarged HDR	23	6	0.7	173	2,9	6.25
D	II-3	F	8 ds	ND	Enlarged HDR	81	3.5	ND	135	ND	15.0
	II-4	M	7 ds	6.4	Enlarged HDR	890	1.15	0.09	83	1,27	11.0
E	II-1	M	10 ds	9.6	Enlarged HDR	190	6.2	1	205	1	8.4
F	II-2	M	3.4 yrs/ re-evaluation at 5.3 yrs	9.7	Enlarged HDR	23.5	5.3	0.75	231	1	3.3
G	II-1	M	14 ds	ND	Enlarged HDR	150	ND	0.44	ND	0.2	6.9
	II-2	F	Antenatal diagnosis/re-evaluation on day 1	Antenatal ultrasound	ND	87.4	ND	0.93	ND	0.2	9.2
Reference range [*,**]				<7 ds: 1.62 ± 0.41, 7–30 ds: 0.84 ± 0.38, < 6 yrs: 2.00, 6–11 yrs: 2.7 ± 0.8, 11–16 yrs: 7.0 ± 2.0, > 16 yrs: 11.6 ± 4.4		<15 ds: 0.64–10.50, < 30 ds: 0.44–8.80, < 1 yr: 0.90–7.70, > 1 yr: 0.40–4.00	<30 ds: 8.0–17.0, < 1 yr: 7.2–15.6, > 1 yr: 5.5–12.8	<1 yr: 0.9–2.6, > 1 yr: 0.9–2.3	<30 ds: 117–263, < 1 yr: 105–245, > 1 yr: 80–200	<15 ds: 28.30–173.00, < 30 ds: 6.44–82.80, < 1 yr: 6.00–80.00, > 1 yr: 1.40–78.50	

In A:III-1, A:III-2 and F:II-2 patients the imaging and laboratory testing correspond to the re-evaluation.
M, male; F, female; ND, Not determined; yr, year; yrs, years, ds; days; HDR, Homogeneous Distribution of Radiotracer.
Thyroid volume was calculated by multiplication of length, breadth and depth and a corrective factor (0.52) for each lobe.
The imaging and laboratory testing reflect the hormonal situation before adjustment L-T4 of substitution.
[*] Reference range for family F: TSH (mIU/L), 0.5–5; TT_4 (μg/dl), 6–14; FT_4 (ng/dl), 0.80–2.20; TT_3 (ng/dl), 80–220; TG (ng/ml), <1 year: 11.5–98, >1 year: 2–30.
[**] Reference range for family G: TSH (mIU/L), 0.35–5; FT_4 (ng/dl), 0.70–1.71; TG (ng/ml), <1.

2.1.3. Family C

2.1.3.1. Patient II-4. This patient is the fourth child of an unrelated couple diagnosed at 9 days of life through the neonatal screening program. She was born at term by a spontaneous vaginal delivery. There were no complications during the pregnancy. She had a goiter. Follow-up showed normal clinical growth and development of the child. At present, she attends primary school without difficulties.

2.1.4. Family D

2.1.4.1. Patient II-3. This patient is the third child of a non-consanguineous couple, diagnosed with congenital hypothyroidism at 8 days of life through the neonatal screening program. She was born at term after a noncomplicated pregnancy, delivered by cesarean section. Their thyroid volume was considerable on scintigraphic examination by means of ^{99}Tc (Table 1).

2.1.4.2. Patient II-4. Patient II-4, patient II-3's brother, diagnosed at 7 days of life through the neonatal screening program. He was born from an uneventful pregnancy and normal delivery. The initial pediatric evaluation showed goiter, macroglossia, umbilical hernia and dry skin. His growth and development were normal. He received a standard education and her mental function appeared to be normal.

2.1.5. Family E

2.1.5.1. Patient II-1. This patient is the first child of a non-consanguineous couple diagnosed at 10 days of life, through the neonatal screening program. He had a goiter. His two grandmothers are under hypothyroidism therapy. The boy grew and developed normally.

2.1.6. Family F

2.1.6.1. Patient II-2. Patient II-2 is the second child of a non-consanguineous couple and was referred at 3.4 years of age because of goiter. He was born at term after a noncomplicated pregnancy, delivered by cesarean section. His neonatal screening based on filter paper TSH determination was informed as normal. At consultation he was clinically euthyroid with a goiter. Blood analysis indicated elevated TSH with low TT_4. Treatment with L-T_4 was started at age 3.5. TSH was normalized and the patient grew normally. Thyroid size remained stable. At 5.3 years his thyroid function was re-evaluated after a month of treatment withdrawal (Table 1). Potassium perchlorate discharge test was negative (<10%). Treatment was reinitiated and his compliance was not good. He grew up normally and is starting puberty (11.1 years). He is mildly retarded but school performance is normal with extra support. His mother (I-2) had a goiter palpable in orthoptic position of 35 g, with normal biochemical thyroid parameters (TSH: 0.64 mU/L, TT_4: 8.6 g/dl, FT_4: 1.3 ng/dl, TT_3: 122 ng/dl, 9.6 ng/ml), negative anti-TG, anti-TPO and anti-rTSH antibodies and without symptoms compatible with hypothyroidism.

2.1.7. Family G

2.1.7.1. Patient II-1. Patient II-1 was diagnosed with congenital hypothyroidism on neonatal screening tests. He was born at term as the first child of a healthy, non-consanguineous couple following an uneventful pregnancy. Routine antenatal ultrasound scans were normal. Parents each had healthy children from previous relationships. There was no clinical evidence of goiter and there were no other abnormal findings apart from very dry skin. His confirmatory blood tests at 14 days of age showed elevated TSH with low FT_4. ^{99}Tc scintigraphy at three weeks of age revealed a mildly

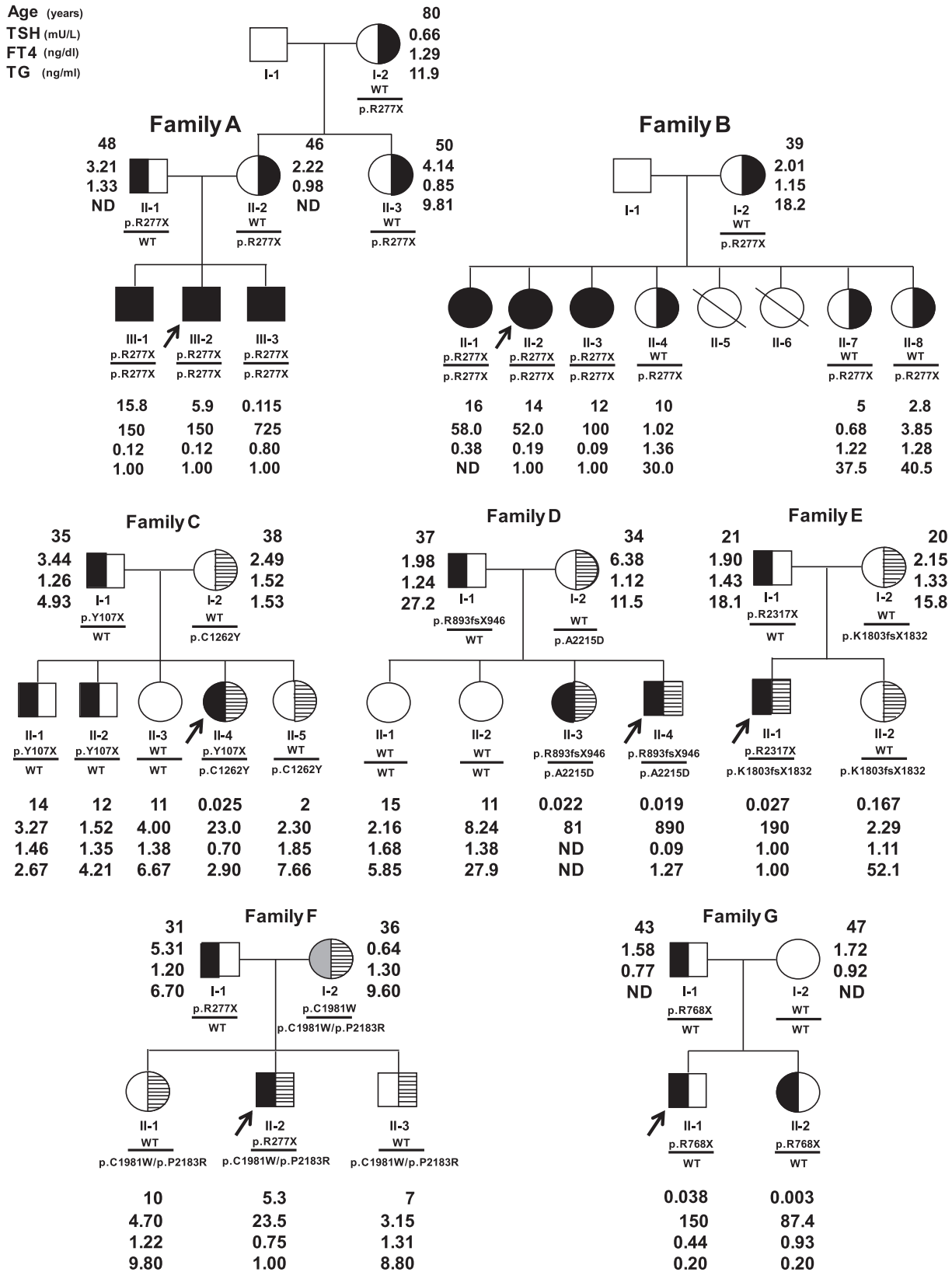

Fig. 1. Pedigrees showing the genotype and thyroid function test results in families A–G. Squares represent males and circles females; arrows indicate the proband in each family. All data are aligned with each individual symbol on the pedigree. Filled symbols denote affected individuals and half-filled symbols, unaffected heterozygote individuals. The black solid symbol indicates the p.R277X (families A, B and F), p.Y107X (family C), p.R893fsX946 (family D), p.R2317X (family E) or p.R768X (family G) mutant alleles; the hatched symbol, p.C1262Y (family C), p.A2215D (family D), p.K1803fsX1833 (family E) or p.C1981W-p.P2183R (family F) mutant alleles and the gray solid symbol the p.C1981W (family F) mutant allele. The grandfather I-1 from family A and father I-1 from family B were not availables for analysis. Consequently, in II-4, II-7 and II-8 from family B we were not able to determine if the mutation was inherited from the father or the mother. Note that patient II-1 and II-2 from family G did not show a second inactivating mutation. Diagonal lines denote deceased individuals. ND, Not Determined. The ages indicated correspond to the age when thyroid tests were performed. See Table 1 for reference ranges of thyroid function test.

enlarged thyroid gland with a uniform distribution of activity (Table 1). Follow-up showed normal growth. He is in mainstream school but needs help with some subjects and is being assessed for dyslexia.

2.1.7.2. Patient II-2. In patient II-2, patient II-1's sister, a large fetal goiter was diagnosed by ultrasound at the 20th weeks of gestation. Escalating doses of L-T_4 were given by injection into the amniotic fluid. Therapy was started with 100 and 200 g at 23 and 25 + 5 weeks gestation, respectively. An umbilical vein blood sampling was performed at 28 weeks, confirming fetal hypothyroidism with elevated serum TSH (14 mIU/L) and low FT_4 (0.31 ng/dl). The subsequent doses were: 28 + 2: 300 g; 30 + 2: 500 g; 32 + 1: 650 g; 34 + 2, 750 g. The baby girl was born by elective caesarian section at 36 weeks gestation and was intubated on perineum because of concern that the goiter would compromise the airway. However, she was extubated into air at 8 h of age, was breast fed from birth and there were no other complications. On day 1 her thyroid function showed an elevated TSH with a FT_4 at the lower limit of normal range (Table 1). She showed good growth and development.

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).

2.3. Genomic PCR amplification

Genomic DNA was isolated from peripheral blood leucocytes by using the cetyltrimethylammonium bromide (CTAB) method and stored at −20 °C until analyzed. The 180 bp of the promoter 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 reported elsewhere (Gutnisky et al., 2004).

2.4. Screening of mutations in the TG gene by AlwNI, Taq I and Hae II restriction analysis

AlwNI (New England Biolabs, Ipswich, MA, USA), TaqI (NewEngland Biolabs) and Hae II (NewEngland Biolabs) endonucleases were used to screen for the presence of the c.886C>T [p.R277X, exon 7] (van de Graaf et al., 1999), c.4588C>T [p.R1511X, exon 22] (Targovnik et al., 1993), and c.6725G>A [p.R2223H, exon 38] (Caron et al., 2003) inactivating mutations, respectively in PCR fragments. AlwNI, Taq I and Hae II restriction fragments were analyzed in a 2.5% agarose gel.

2.5. Single-strand conformation polymorphism analysis (SSCP)

TG PCR fragments were analyzed by SSCP as detailed previously (Machiavelli et al., 2010). The gel matrix contained 8% polyacrylamide (29:1) without glycerol. Samples were electrophoresed for 24 h at a constant temperature (4 °C). DNA was visualized by silver-staining according to standard procedures.

2.6. 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).

2.7. Cloning of wild-type and mutant alleles

The amplified PCR fragments corresponding to exon 10 from patient II-4 of family D and exon 28 from patient II-4 of family C was T-A cloned into pGEM-T Easy vector (Promega, Madison, WI, USA). DNA sequencing was performed as described above from wild type and mutant allele clones using SP6 and T7 vector primers.

2.8. Identification of c.6000C>G [p.C1981W] mutation by SSCP analysis

SSCP was used to screen for the presence of c.6000C>G [p.C1981W] mutation in healthy unrelated individuals. PCR to amplify exon 33 and SSCP conditions were performed as described above.

2.9. Identification of c.6605C>G [p.P2183R] mutation by BaeGI restriction analysis

BaeGI (New England Biolabs, Ipswich, MA) restriction endonuclease were used to screen for the presence of the c.6605C>G [p.P2183R, exon 38] mutation in healthy unrelated individuals. PCR products (414 bp) were digested with BaeGI according to manufacturer specifications. The mutation destroys a BaeGI recognition site. BaeGI restriction fragments were analyzed in a 3% agarose gel. Digestion of the wild-type allele results in two fragments of 135 and 279 bp.

2.10. In silico prediction analysis

Amino acid sequence homology between several TG species was compared using the MegAlign software program (DNASTAR, Hauser University of California-SF). Human TG was submitted to the Jpred 3 (http://www.compbio.dundee.ac.uk/www-jpred/) internet site for in silico analysis of the protein secondary structure prediction. This algorithm provided a three-state (α-helix, β-strand and coil) prediction of secondary structure at 81.5% accuracy. For protein secondary structure comparison the position-specific scoring matrix output coming from Jpred 3 web system was performed.

2.11. Nucleotide and amino acid nomenclatures

The nucleotide position in human TG mRNA was 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 (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 AlwNI, Taq I and Hae II restriction analysis

AlwNI, Taq I and Hae II endonucleases were used to screen for the presence of the c.886C>T [p.R277X, exon 7], c.4588C>T [p.R1511X, exon 22], and c.6725G>A [p.R2223H, exon 38] mutations, respectively, in patients III-1, III-2 and III-3 of family A, II-1, II-2 and II-3 of family B, II-2 of family F and II-1 and II-2 of family G. AlwNI restriction analysis showed that the three affected subjects of family A and the three ones of family B are homozygous for the mutation c.886C>T, subject II-2 of family F is heterozygous for this mutation, whereas both siblings of family G contain wild type alleles (Fig. 1). Sequencing of the exon 7 from the 7 samples, showing the mutated patterns, confirms the presence of c.886C>T

mutation. TaqI and HaeII restriction analysis displays only wild type alleles in all patients.

3.2. Screening of mutations in the TG gene by SSCP

All of 48 exons and exon/intron boundaries of the TG gene of three patients from three unrelated families (family C, II-4; family D, II-4; family E, II-1) were PCR amplified and then screened by SSCP.

We identified four different patterns of migration that were not detected in the healthy controls. Sequence analysis of the samples showing the aberrant conformers revealed two known and two novel mutations, all in heterozygous state. One of them, detected in index patient II-4 of family C, was a novel c.3842G>A transition in exon 17 that results in the replacement of cysteine by tyrosine at amino acid position 1262 [p.C1262Y] (Figs. 1 and 2). The second mutation, characterized in index patient II-1 of family E, was a novel single adenine deletion at nucleotide position 5466 (c.5466delA) in exon 28 resulting in a frameshift at amino acid 1803 with a putative premature stop codon at amino acid 1832 in the exon 30 (p.K1803fsX1832) (Figs. 1 and 3). The c.5466delA mutation was confirmed by cloning in pGEM-T Easy vector and sequencing of both wild type and mutant alleles. The third mutation, detected in index patient II-4 of family D was previously reported as a missense mutation due to a cytosine to adenine transversion, located at nucleotide 6701 in exon 38 (c.6701C>A), which replaces the wild-type alanine at codon 2215 by an aspartic acid [p.A2215D] (Caputo et al., 2007a; Pardo et al., 2008, 2009; Machiavelli et al., 2010) (Fig. 1). The fourth mutation, identified also in index patient II-1 of family E, was a documented cytosine to thymine transition at nucleotide position 7006 (c.7006C>T) in exon 40 which replaces an arginine residue at position 2317 by a stop codon [p.R2317X] (Machiavelli et al., 2010; Liu et al., 2012) (Fig. 1).

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

To identify the second deleterious TG mutation from the three unrelated patients that carry one TG mutation detected by AlwNI restriction analysis (patient II-2 of family F, see above) or by SSCP method (patient II-4 of family C and patient II-4 of family D, see above), 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. The index patient II-1 of family G was also screened by 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 this group of patients. In addition of five mutations which have been detected in the screening by AlwNI analysis or by SSCP method, five novel mutations were identified by direct sequencing. One of them, detected in index patient II-4 of family C, was a cytosine to adenine transversion at nucleotide position 378 (c.378C>A) in exon 4, which replaces a tyrosine residue at position 107 by a premature stop codon [p.Y107X] (Figs. 1 and 2). The second mutation, identified in index patient II-1 of family G, was a cytosine to thymine transition at nucleotide position 2359 in exon 10. Instead of encoding for an arginine residue at position 768, the triplet harboring the mutation encodes a stop codon (p.R768X) (Figs. 1 and 2). Recently, during the review of this paper, De Marco et al. described in homozygous state the p.R768X mutation in two sisters affected with congenital hipothyroidism (De Marco et al., 2012). The third mutation, found in index patient II-4 of family D, was a single guanine deletion at nucleotide position 2736 (c.2736delG) in exon 10 resulting in a frameshift at amino acid 893 with a putative premature stop codon at amino acid 946 in exon 11 (p.R893fsX946) (Figs. 1 and 4). The c.2736delG mutation was confirmed by cloning in pGEM-T Easy vector and sequencing of both wild type and mutant alleles. The fourth mutation, characterized in index patient II-2 of family F was a missense mutation in exon 33, where a cytosine to guanine transversion at nucleotide position 6000 (c.6000C>G) produces the substitution of a cysteine for tryptophan at codon 1981 [p.C1981W] (Figs. 1 and 2). The latter mutation, identified also in index patient II-2 of family F, was a cytosine to guanine transversion at nucleotide position 6605 (c.6605C>G) in exon 38 that results in the replacement of proline for arginine at amino acid position 2183 [p.P2183R] (Figs. 1 and 2).

3.4. Segregation analysis of the mutations in TG gene

In family A, patients III-1, III-2 and III-3 were homozygous for c.886C>T [p.R277X]. Analysis by AlwNI restriction and sequencing of their parents, grandmother and aunt showed that they are healthy heterozygous carriers of the mutation (Fig. 1).

In family B, patients II-1, II, 2 and II-3 were also homozygous for c.886C>T. Screening of mutation by AlwNI endonuclease and subsequent sequencing revealed that their mother and three sisters II-4, II-7 and II-8 are healthy heterozygous carriers of this mutation (Fig. 1). Unfortunately, the father I-1 was not available for segregation analysis. Consequently, in II-4, II-7 and II-8, we were not able to determine if the mutation was inherited from the father or the mother.

The p.R277X mutation has been described previously in heterozygosity or homozygosity in Argentinean, Brazilian, Galician and French populations (van de Graaf et al., 1999; Gutnisky et al., 2004; Rivolta et al., 2005; Caputo et al., 2007a,b; Pardo et al., 2009; Machiavelli et al., 2010; Peteiro-Gonzalez et al., 2010; Citterio et al., 2011). To discriminate between a de novo recurrence of the p.R277X mutations and a founder effect in Argentinean patients, we compared the haplotypes identified in the index patient III-2 of family A with the haplotypes from the index patient II-2 of the family B. 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 (van de Graaf et al., 2001; Rivolta et al., 2005). The presence of exonic SNPs was evaluated by sequence analysis. The SNP results showed that the two individuals affected are homozygous for the same combinations of polymorphisms (Fig. 5). This is a strong indication that the p.R277X alleles in both families might be derived from a common ancestral chromosome. However, comparative analysis between the haplotype segregation with the mutation p.R277X from families A and B and previously reported Argentinean RM and ME patients (Rivolta et al., 2005; Caputo et al., 2007a) with the same mutation also in homozygous state, showed one difference with patient RM (p.R1980W) and three differences with the patient ME (p.R1980W, p.W2482R and p.Y2621Y) (Fig. 5). These findings confirm that it is very likely that the p.R277X mutation is also an independent mutational event in Argentinean population.

In family C, index patient II-4 was a compound heterozygous for c.378C>A/c.3842G>A [p.Y107X/p.C1262Y] who inherited a copy of c.378C>A transversion from his father and a copy of c.3842G>A transition from his mother (Fig. 1). Two brothers, II-1 and II-2, were found to be heterozygous healthy carriers of c.378C>A, whereas an unaffected sister II-5 was carrying the c.3842G>A mutation (Fig. 1).

In family D, index patient II-4 was a compound heterozygous for c.2736delG/c.6701C>A [p.R893fsX946/p.A2215D]. Sequence analysis disclosed the father as the heterozygous carrier of c.2736delG and the mother as the heterozygous carrier of c.6701C>A, whereas the affected sister II-3 was a compound heterozygous for both aberrant variants (Fig. 1).

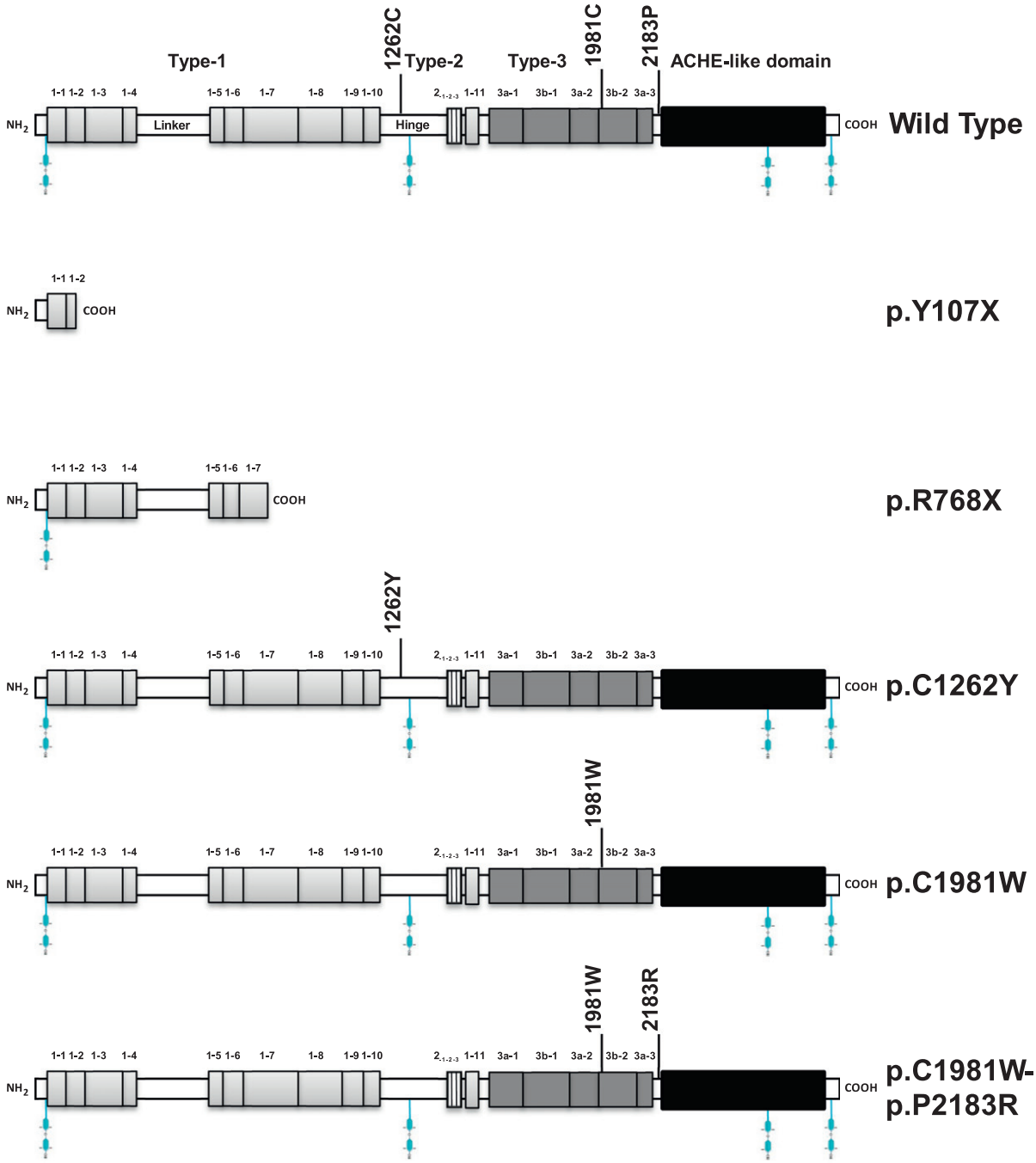

Fig. 2. Schematic representation of the repetitive, acetylcholinesterase homology and hormonogenic domains in the wild-type and putative mutant thyroglobulin proteins (p.Y107X, p.R768X, p.C1262Y, p.C1981W and p.C1981W-p.P2183R). The repetitive units (Types 1, 2 and 3) and the acetylcholinesterase homology domain (ACHE-like domain) are represented by boxes. The positions of T_4 (5, 1291 and 2747) and T_3 (2554) are shown.

In family E, index patient II-1 was a compound heterozygous for c.5466delA/c.7006C>T [p.K1803fsX1832/p.R2317X]. The sequence results demonstrated that II-1 inherited the c.7006C>T from his father and the c.5466delA from his mother (Fig. 1). The healthy sister II-2 was heterozygous for c.5466delA (Fig. 1).

In family F, three variants of sequence, c.886C>T/c.6000C>G/c.6605C>G [p.R277X/p.C1981W/p.P2183R] were identified in index patient II-2 (Figs. 1 and 6). Analysis by direct sequencing of PCR products of exons 7, 33 and 38 from the parents of index patient II-2 showed that c.886C>T was derived from his father and c.6000C>G/c.6605C>G from his mother (Fig. 1). The c.6000C>G mutation was detected in homozygous state in the mother, whereas the c.6605C>G mutation was found in heterozygous state (Fig. 1), indicating that these two variants are in allelic association in one of the chromosomes.

The previously characterized exonic TG SNPs c.4506C>T, c.5995C>T, 6695C>T, c.7408C>T, c.7501T>C, c.7589G>A and c.7920C>T (van de Graaf et al., 2001; Rivolta et al., 2005) were used to determine the allelic distribution in the family of index patient II-2 by DNA sequencing. Specific haplotypes were identified for the c.886C>T and c.6000C>G/c.6605C>G mutated alleles. As shown in Fig. 7, the father (I-1) harbors C, C, C, C, G and T in the SNPs local-

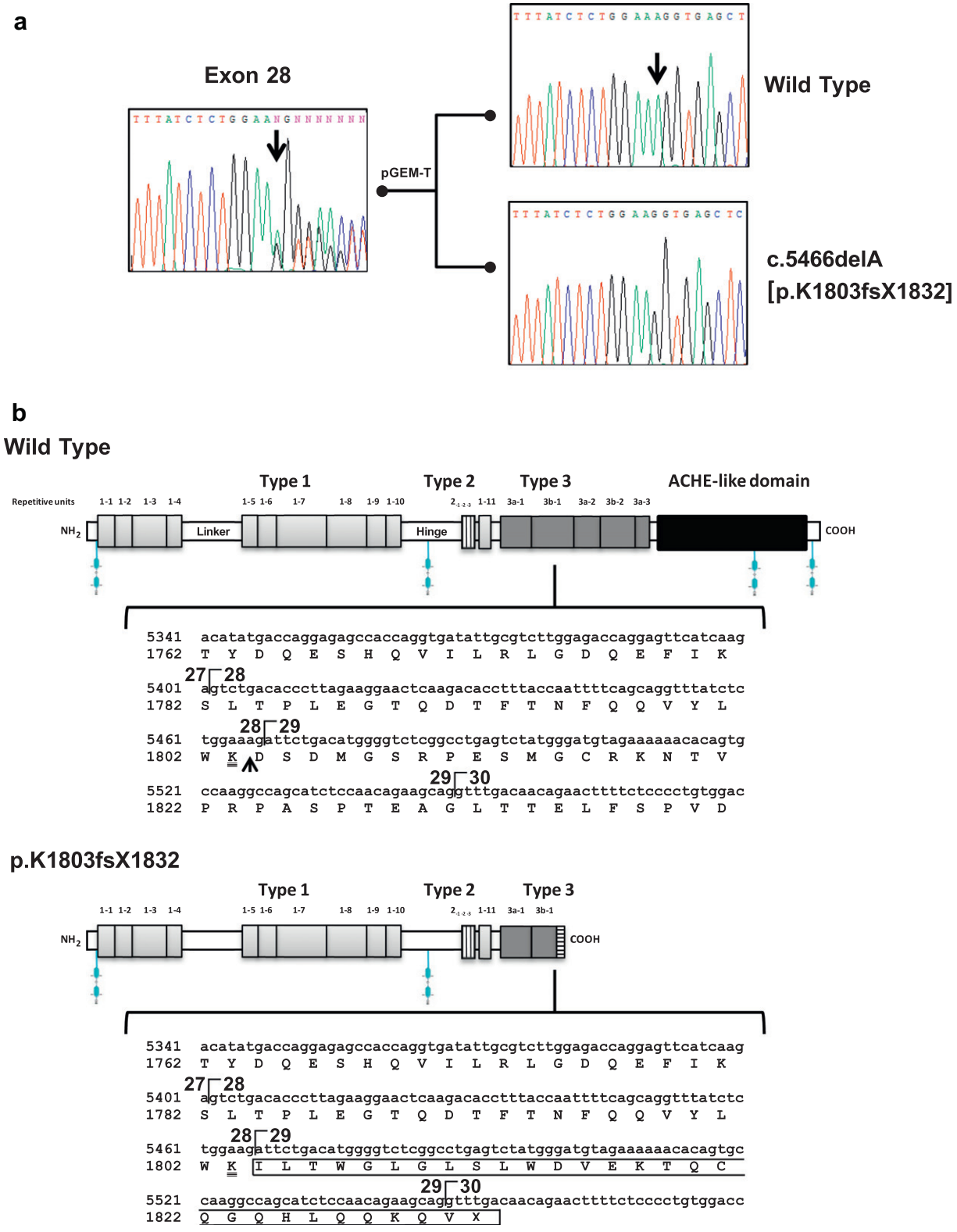

Fig. 3. (a) Sequence analysis of exon 28 from patient II-1 of family E. Partial sequencing chromatograms of PCR product, and wild type and mutated allele cloned into pGEM-T Easy vector are shown. Sense strand is displayed. Arrows denote the position of c.5466delA mutation, single chromatogram peaks indicate homozygosity and two overlapping peaks at the same locus, heterozygosity. (b) Structural organization of the wild-type and putative p.K1803fsX1832 mutant thyroglobulin proteins. The repetitive units of type 1–3 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. The linker and hinge segments are indicated. The partial nucleotide and the deduced amino acid sequences from wild-type and putative c.5466delA mutant are reported below the respective schematic protein diagrams. The nucleotide sequence is given in the upper line, and the amino acid translation (represented by single-letter code) is given below their respective codons. The arrow denotes the positions of the c.5466delA mutation and the resulting frameshift are boxed. The 1803 lysine is unchanged (AAA > AAG) and is underlined. ⌈ indicates exon/exon boundaries and exon numbering is shown.

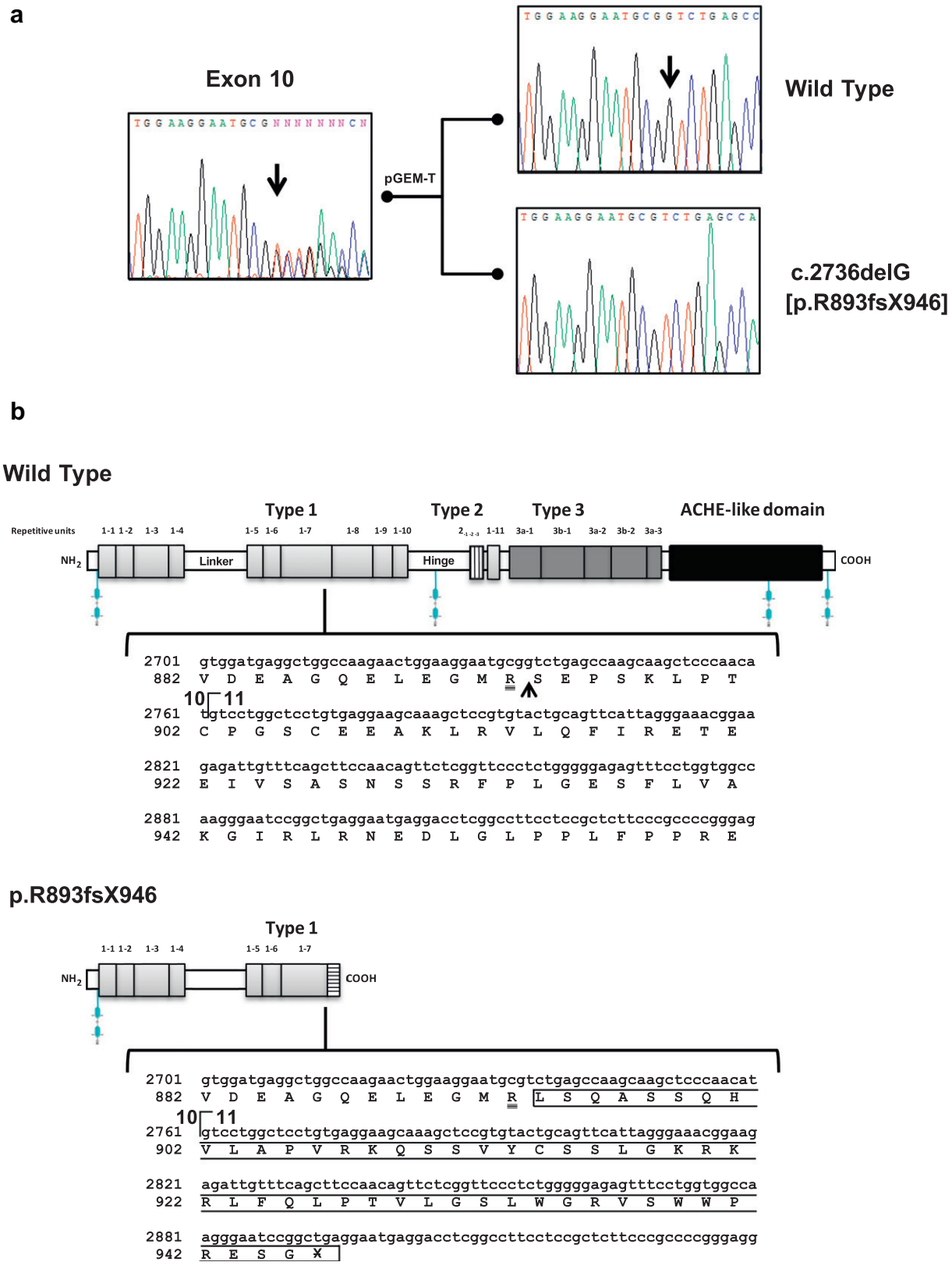

Fig. 4. (a) Sequence analysis of exon 10 from patient II-4 of family D. Partial sequencing chromatograms of PCR product, and wild type and mutant allele cloned into pGEM-T Easy vector are shown. Sense strand is displayed. Arrows denote the position of c.2736delG mutation, single chromatogram peaks indicate homozygosity and two overlapping peaks at the same locus, heterozygosity. (b) Structural organization of the wild-type and putative p.R893fsX946 mutant thyroglobulin proteins. The repetitive units of type 1–3 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. The linker and hinge segments are indicated. The partial nucleotide and the deduced amino acid sequences from wild-type and putative c.2736delG mutant are reported below the respective schematic protein diagrams. The nucleotide sequence is given in the upper line, and the amino acid translation (represented by single-letter code) is given below their respective codons. The arrow denotes the positions of the of c.2736delG mutation and the resulting frameshift are boxed. The 893 arginine is unchanged (CGG > CGT) and is underlined. ⌈ indicates exon/exon boundaries and exon numbering is shown.

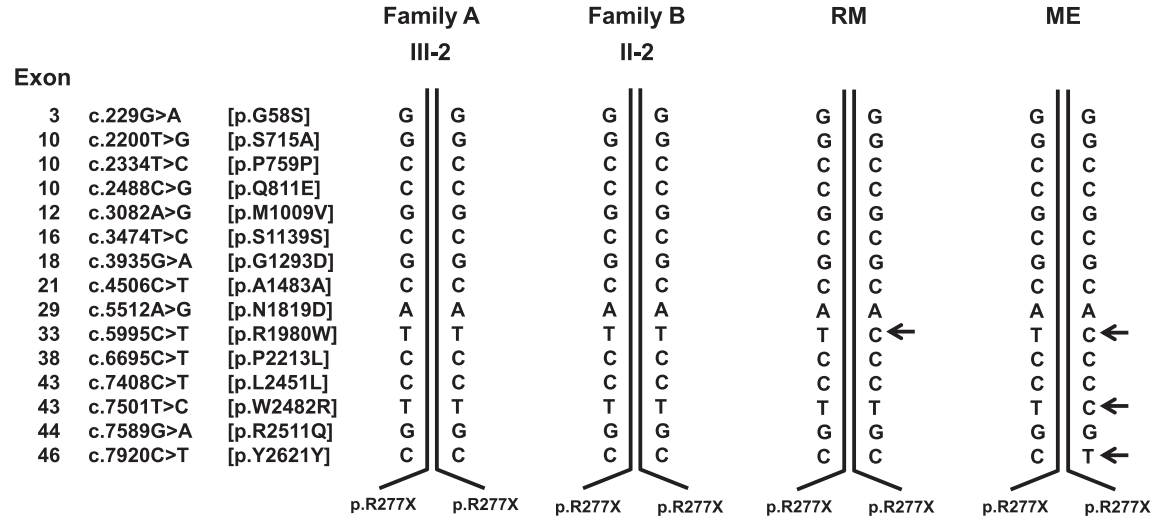

Fig. 5. Comparative haplotype analysis of the patients III-2 (family A), II-2 (family B) RM and ME with the p.R277X mutation in homozygous state, using 15 exonic SNPs. The arrows denote differences between haplotypes. The parents of ME were not available for segregation analysis; consequently both haplotypes in this patient are hypothetical. Note that Patients III-2 (family A) and II-2 (family B) are homozygous for the same combinations of SNPs.

ized in the nucleotide positions 4506, 6695, 7408, 7501, 7589 and 7920, respectively, in the allele associated with the presence of the c.886C>T mutation, whereas the mother (I-2) harbors T, C, C, T, G and C in the same SNPs, in the allele associated with the presence of the c.6000C>G/c.6605C>G mutations. Surprisingly, the genotypes for the polymorphism c.5995C>T and the novel mutation c.6000C>G in exon 33 are contradictory in the family. The father (I-1) is homozygous for cytosine 5995 and the wild-type cytosine 6000, whereas patient II-2 and her mother (I-2) are homozygous for thymine 5995 and the mutated guanine 6000 (Fig. 7). This strongly suggested that the index patient II-2 did not inherit from his father the cytosine 5995 and the wild-type cytosine 6000, indicating a deletion that includes exon 33, in the allele associated with the mutation c.886C>T (Fig. 7). These results were replicated using independent DNA samples. Of the 24 SNP previously identified in the TG coding sequences (van de Graaf et al., 2001; Rivolta et al., 2005), three were heterozygous (c.4506C>T [p.A1483A, exon 21], c.7501T>C [p.W2482R, in exon 43], and c.7920C>T [p.Y2621Y, exon 46]) in patient II-2 (Fig. 6), indicating that the deletion is probably limited to the TG gene, involving exon 33. Haplotype analysis of her sister (II-1) and brother (II-3) revealed that both harbor the maternal mutant allele c.6000C>G/c.6605C>G and the paternal wild-type allele (Fig. 7). In addition, haplotype analysis in the sister (II-1) inferred the presence in this family member of a meiotic recombination event for paternal gametes, which includes the 3' region of the TG gene. The c .7501T>C and c.7920C>T SNPs are informative, II-1 carries C and T from paternal mutant allele instead T and C from the wild-type allele, respectively (Fig. 7).

In family G, segregation analysis by direct sequencing indicated that index patient II-1 and his sister (II-2) inherited c.2359C>T mutation from their father (Fig. 1). However, index patient II-1 did not show an additional inactivating mutation, 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. Major deletions involving one of the TG alleles can be disregarded because of the presence of exonic polymorphisms in the heterozygous state. Of the 24 reported SNPs in the TG coding sequences (van de Graaf et al., 2001; Rivolta et al., 2005) four were heterozygous (c.6695C>G [p.P2213L, exon 38], c.7408C>T [p.L2451L, exon 43], c.7501T>C [p.W2482R, exon 43], and c.7920C>T [p.Y2621Y, exon 46]) in index patient II-1 (Fig. 6).

3.5. Validation of c.3842G>A, c.6000C>G, and c.6605C>G mutations

We ruled out the possibility that the c.3842G>A [p.C1262Y], c.6000C>G [p.C1981W] and c.6605C>G [p.P2183R] mutations could be polymorphisms because they were not detected in 100 chromosomes from the general population by SSCP analysis (c.3842G>A, c.6000C>G) or BaeGI restriction analysis (c.6605C>G).dbSNP blast (http://www.ncbi.nlm.nih.gov/SNP/index.html), Exome Variant Server (NHLBI GO Exome Sequencing Project (ESP), Seattle, WA (URL: http://evs.gs.washington.edu/EVS/) and 1000 Genomes Project (URL: http://browser.1000genomes.org/index.html) analysis revealed that c.3842G>A and c.6000C>G mutations have not been identified as variations in the TG gene. By contrast, the c.6605C>G mutation was available in these data bases with an estimated frequency of 0.999% for the allele C^{6605}, whereas the allele G^{6605} with a frequency of 0.001% is a rare variant of sequence that was found only in heterozygous state.

3.6. Homology and protein secondary structure analysis

Four out of seven novel mutations identified in this study cause a premature stop codon in TG polypeptide coding sequences, resulting in a truncated polypeptide. The deleterious effect of these mutations is obvious. The detrimental effect caused by the other three missense mutations was not so significant. Consequently, the deleterious effect of the p.C1262Y, p.C1981W and p.P2183R mutations was evaluated by assessing the degree of evolutionary conservation of the respective amino acids among several animal wild-type TGs and by protein secondary structure prediction analysis.

Multiple sequence alignment of the human TG with sequences found in the GenBank database, using Clustal method, revealed that wild-type cysteine residues at positions 1262 and 1981 are strictly conserved in all TG species analyzed (Fig. 8a), whereas the wild-type proline residue at position 2183 is conserved in bovine and is substituted in rat and mouse by histidine and arginine, respectively (Fig. 8a).

Predictive analysis of the protein secondary structure spanning residues 1055–1315 showed that the mutation p.C1262Y induces a significant rearrangement. The extension of two β-strand segments was increased (^{1210}IL^{1211} → ^{1209}TILC^{1212}, ^{1244}I → ^{1243}LIC^{1245}), one β-

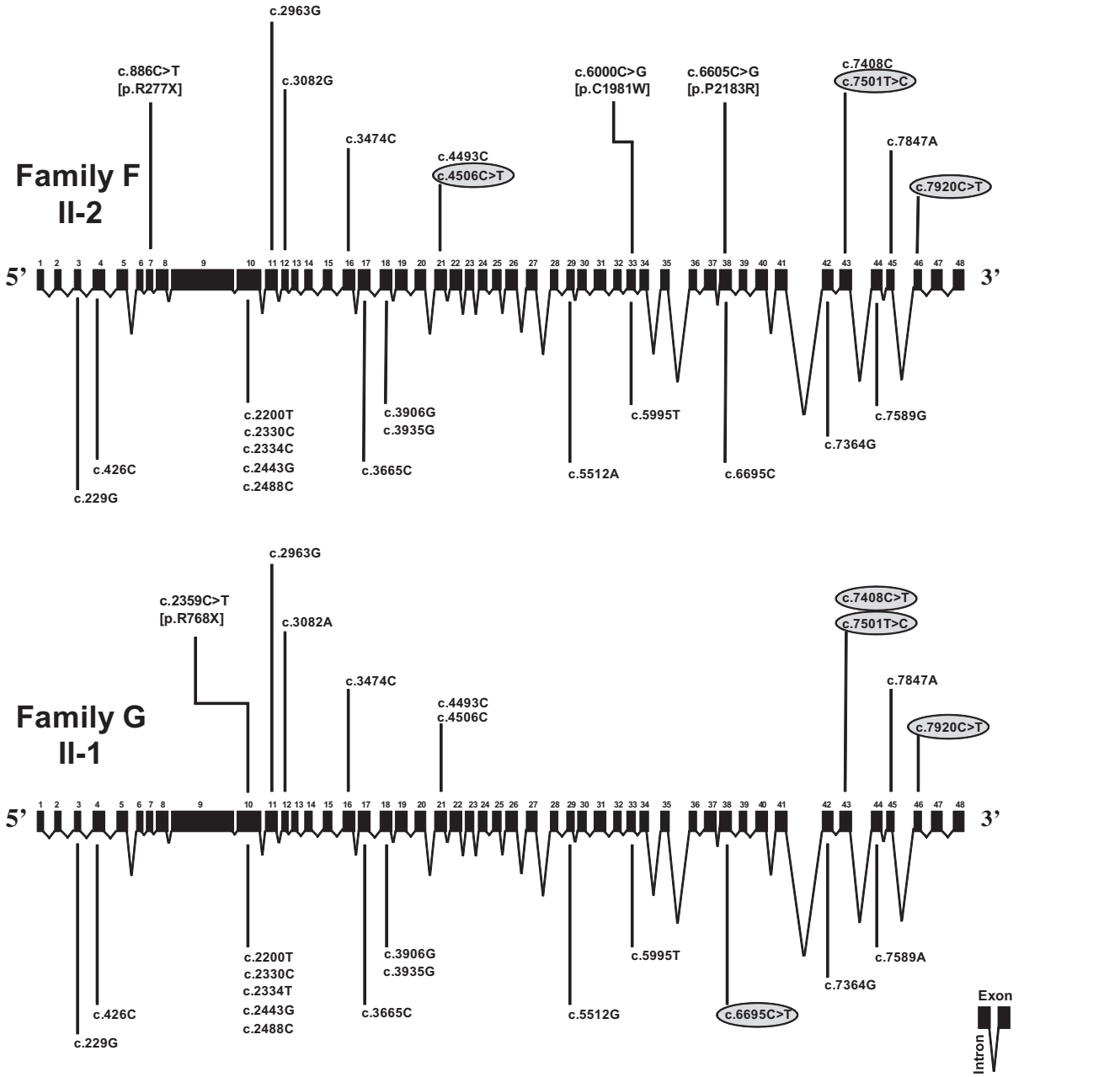

Fig. 6. Identification of c.886C>T (p.R277X), c.6000C>G (p.C1981W) and c.6605C>G (p.P2183R) mutations in patient II-2 of family F and c.2359C>T (p.R768X) mutation in patient II-1 of family G, and schematic respresentation of the exon/intron organization of the thyroglobulin gene with indication of positions of the exonic single nucleotide polymorphisms (SNPs). Note the difference between scales used for introns and exons. Orientation is given according to the mRNA structure. The gray areas indicate the heterozygous SNPs.

strand was eliminated (^{1251}RWE^{1253}) and one α-helix was reduced (^{1291}YAGLLQTFQVFILDELTA^{1308} → ^{1294}LLQTFQVFILDELTA^{1308}) (data not shown).

To explain the cooperative effect of the two missense mutations (p.C1981W, p.P2183R) in index patient II-2 of family F, we performed an in silico study on secondary structure of TG mutants. The analysis was focused on the region between residues 1973 and 2242 and five variants were compared: wild type 1 (containing the polymorphic residues R^{1980} and P^{2213}, which corresponds to paternal wild-type allele), wild type 2 (containing the polymorphic residues W^{1980} and P^{2213} which will be considered as a control wild-type allele), mutant p.C1981W (maternal mutant allele), mutant p.P2183R (which will be considered as a control mutant allele), and double mutant p.C1981W/p.P2183R (maternal mutant allele) (Fig. 8b). The predicted structure of wild type 1 was characterized by 14 β-strand segments spanning residues 1990–1994, 2002–2008, 2014–2016, 2024–2028, 2035–2038, 2082–2084, 2092–2097, 2121–2126, 2132–2138, 2153–2157, 2164–2165, 2181–2184, 2189–2198, and 2206–2209 of polypeptide chain and three α-helices in the residues 2070–2072, 2098–2100 and 2104–2115 (Fig. 8b). In wild type II, the three α-helices were unmodified and the extension of β-sheet segment was slightly reduced between 2133 and 2138 (VRCMFY), followed by a slight increase of the β-sheet between 2152 and 2157 (NCRLLL) (Fig. 8b). In p.C1981W mutant, the three α-helices and all β-sheet segments remained unmodified relative to control wild type 2, whereas p.P2183R induced a slight reduction of β-sheet between residues 2153–2157 (CRLLL) and a conversion from β-sheet to α-helix in the segment spanning residues 2164–2165 (IY) (Fig. 8b). The p.C1981W/p.P2183R double mutant displayed the major structural

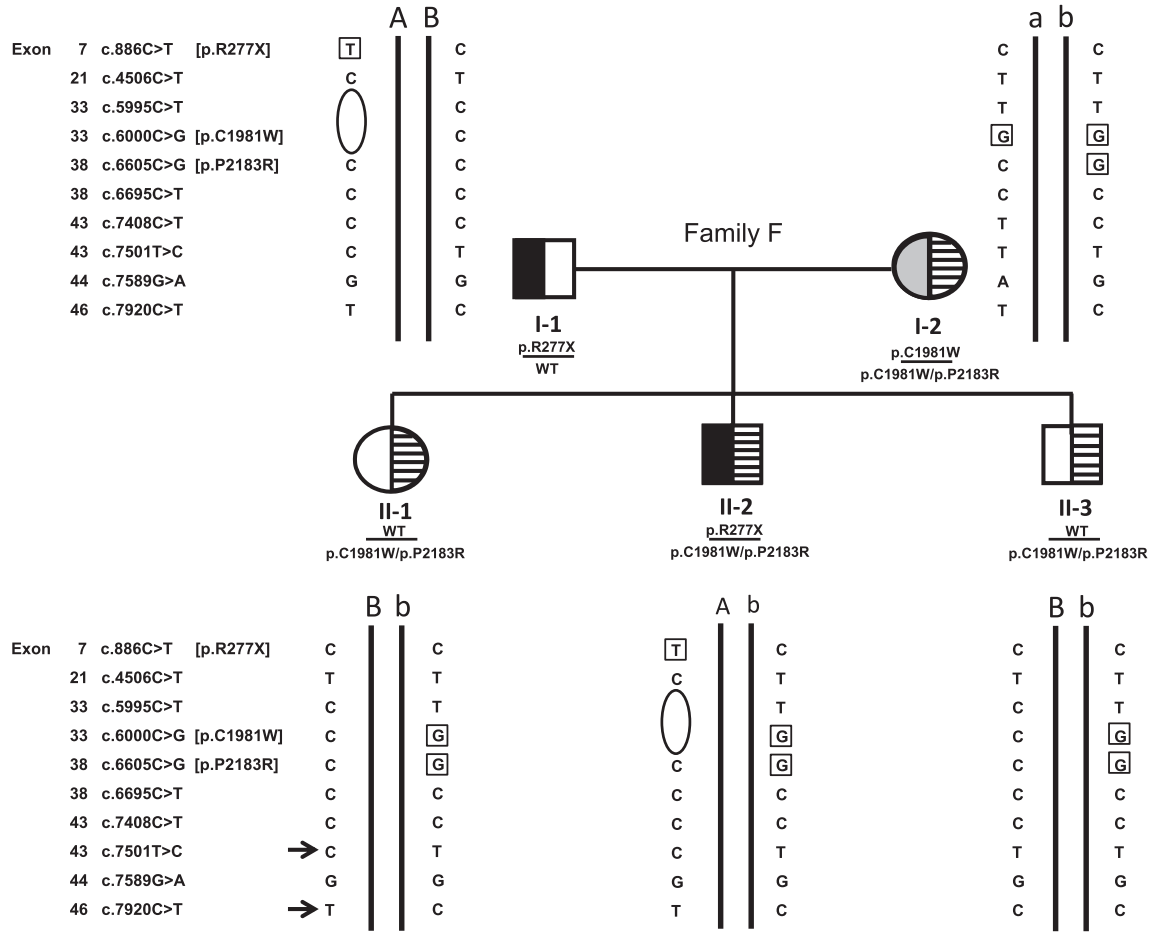

Fig. 7. Pedigree of family F showing the pattern of inheritance of the mutant thyroglobulin alleles. All data are aligned with each individual symbol on the pedigree. Note that patient II-2 has inherited one copy of the p.R277X from his father (I-1) and one copy of the p.C1981W-p.P2183R mutations from his mother (I-2), whereas his sister II-1 and brother II-3 have inherited one copy of the wild-type allele from their father and one copy of the p.C1981W-p.P2183R mutations from their mother. Squares represent males and circles females. Filled symbols denote affected individuals and half-filled symbols, unaffected heterozygote individuals. Black solid symbols indicate the p.R277X mutated allele, the hatched symbol the p.C1981W-p.P2183R and the gray solid symbol the p.C1981W mutant allele. A recombination event has been recorded for the unaffected II-1 (arrows). ○ represents the hypothetical deleted region.

modifications relative to control wild type 2. In addition to significant secondary structure rearrangement caused by the presence of the p.P2183R mutation was predicted in p.C1981W/p.P2183R double mutant that the extension of β-sheet and α-helix segments was slightly reduced between residues 2024–2027 (WRIL) and 2070–2071 (TA), respectively (Fig. 8b). On the contrary, the β-sheet was slightly increased in the segment spanning residues 2035–2039 (EVHTY).

4. Discussion

In the present study we demonstrated that in all the affected individuals the phenotype was associated with inactivating mutations in the TG gene. The patients were homozygous or compound heterozygous and the parents were carriers of TG mutations. Ten mutations have been identified and characterized in the present study: four nonsense mutations (c.378C>A [p.Y107X], c.886C>T [p.R277X], c.2359C>T [p.R768X] and c.7006C>T [p.R2317X]), two single nucleotide deletions (c.2736delG [p.R893fsX946] and c.5466delA [p.K1803fsX1832]), and four missense mutations (c.3842G>A [p.C1262Y], c.6000C>G [p.C1981W], c.6605C>G [p.P2183R] and c.6701C>A [p.A2215D]). The p.R277X, p.A2215D and p.R2317X mutations were previously reported (van de Graaf et al., 1999; Gutnisky et al., 2004; Rivolta et al., 2005; Caputo et al., 2007a,b; Pardo et al., 2008, 2009; Machiavelli et al., 2010; Peteiro-Gonzalez et al., 2010; Citterio et al., 2011; Liu et al., 2012) and the remaining seven are novel.

The misfolded TGs described here, due to truncated proteins (p.Y107X, p.R277X, p.R768X, p.R2317X, p.R893fsX946 and p.K1803fsX1832), missense mutations located in ACHE-like domain (p.A2215D) or replacement of cysteine residues (p.C1262Y and p.C1981W) may cause TG retention in the ER and premature degradation as seen in other ER storage diseases (Targovnik, 2012).

ACHE-like domain is essential for normal conformational maturation and intracellular transport of TG to the site of its iodination and 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). It is well documented that truncated TG comprising only regions I, II, and III and devoid of the ACHE like domain is blocked within the ER, making it incompetent for cellular export (Lee et al., 2008). Our data are consistent with these reported findings. p.Y107X, p.R277X, p.R768X and p.R893fsX946 mutant comprise only a part of region I (Figs. 2 and 4b), p.K1803fsX1832 includes regions I, II and only a part of region III (Fig. 3b), while p.R2317X comprises regions I, II, III and only part of ACHE-like domain. It is highly likely that hydrolysis of limited amounts of TG that escapes from this retention is a possible mechanism providing a minimum amount of thyroid hormones, as observed in most

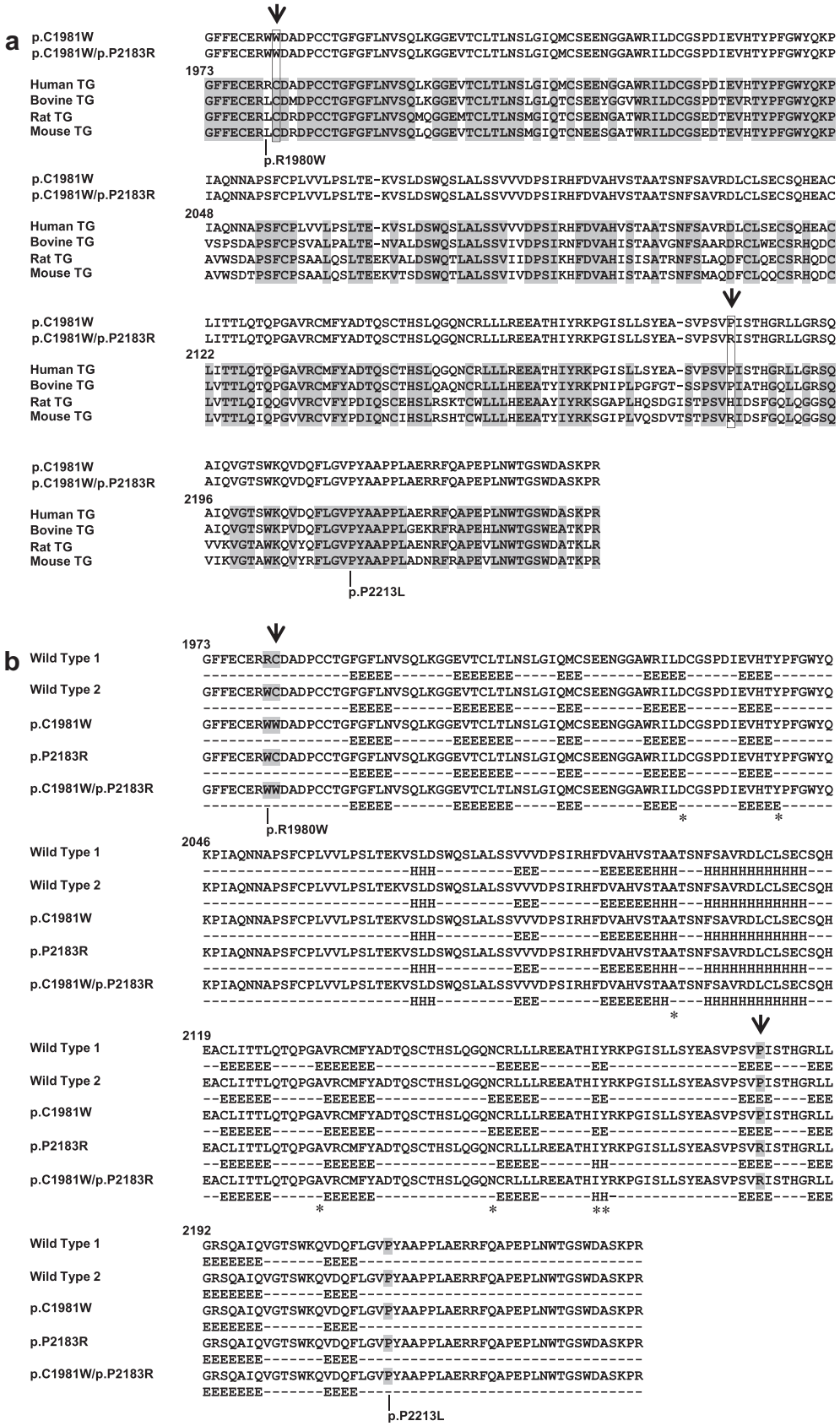

Fig. 8. Homology and protein secondary structure. (a) Partial protein alignment of the human, bovine, rat and mouse. Completely conserved residues are indicated in gray. (b) Protein secondary structure analysis. Abbreviations are E, -sheet; H, helix; -, turn. Asterisks mark the differences in the protein secondary structure. The amino acids are indicated by the single-letter code and the positions of the p.R1980W and p.P2213L SNPs are shown. The arrows denote the positions of the missense mutations (p.C1981W and p.P2183R). The amino acid sequences are based on the GenBank/EMBL/DDBJ protein data base: Homo Sapiens TG (P01266); Bos Taurus TG (P01267); Rattus Norvegicus TG (P06882) and Mus Musculus TG (008710).

cases of defective TG synthesis. In addition to ER storage diseases as a pathophysiological mechanism in the generation of congenital hypothyroidism, these truncated mutants have an impairment in thyroid hormone synthesis. Lacks all the carboxyl-terminal hormonogenic sites (positions: 2554 and 2774). However, all except for p.Y107X are sufficient for the synthesis of T_4 in the N-terminal domain (Targovnik, 2012).

In family F, the segregation analysis showed a very complex genotype for index patient II-2 (Fig. 7), the paternal mutant allele harbors a p.R277X mutation associated with a hypothetical deletion that would involve exon 33, whereas the maternal mutant allele harbors two missense mutations, p.C1981W and p.P2183R. The p.C1981W was detected in homozygous state and p.P2183R was found in heterozygous state. The wild type C^{1981} residue is strictly conserved in all TG species analyzed (Fig. 8a) and the p.C1981W mutation was not detected in the control population. Both events predict a pathogenic mutation. However, the p.C1981W does not cause a significant rearrangement in the protein secondary structure (Fig. 8b). On the contrary, P^{2183} is not conserved (Fig. 8a) and the p.P2183R mutation was detected in the control population with a frequency of 0.001%, two facts indicative of a rare sequence variation. Nevertheless, p.P2183R modifies the protein secondary structure and the double mutant p.C1981W/p.P2183R results in a major structural modification (Fig. 8b). To complete this scenario we observed that his mother has goiter without clinical and biochemical expression of hypothyroidism. It is thus reasonable to believe that the biallelic p.C1981W mutation by itself or associated with the p.P2183R mutation in one allele is not sufficient to produce significant hypothyroidism. Consequently, p.C1981W-p.P2183R double-mutation allele needs a second allele carrying an inactivating mutation, such as p.R277X, to develop the disease, as observed in index patient II-2.

In family G, p.R768X was found in one allele, whereas the mutation in the other allele was not detected. It is unclear whether the phenotype is caused by a monoallelic defect. It is likely that the apparent absence of a second mutation could be explained by technical limitations of the direct sequencing analysis. Similarly, we previously identified three patients from two unrelated families with congenital hypothyroidism that had a single p.R277X mutated allele (Machiavelli et al., 2010). The complete deletion of one of the TG alleles can be disregarded in the present study because of the presence of exonic SNP in the heterozygous state (Fig. 6). However, our analysis does not exclude micro deletions involving one or several exons or mutations in distant regulatory regions of the TG gene. Another possibility is that intronic splicing enhancers and/or suppressors at appreciable distances from exon/intron junctions may have been excluded from our analysis. Interestingly, in this family we also observe that the patient II-1 showed a mildly enlarged thyroid gland after birth, whereas his sister, patient II-2 presented a large fetal goiter with the same genotype. The reason for this phenomenon remains to be elucidated. It is possible that these differences are related to uncommon genetic factors, the degree of sensitivity to the TSH, the ability of the goitrous tissues to adapt to the pathological condition and the effect of environmental factors, such as the availability of sufficient iodine.

In conclusion we have identified and characterized seven novel mutations and three previously reported mutations of the TG gene in 13 patients from 7 non-consanguineous families. This study contributes to the understanding of the molecular mechanisms involved in the development of dyshormonogenesis by defective intracellular trafficking of misfolded TG.

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