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A Novel Mutation in Intron 11 Donor Splice Site, Responsible of a Rare Genotype in Thyroglobulin Gene by Altering the Pre-mRNA Splicing Process. Cell Expression and Bioinformatic Analysis.

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Abstract

Thyroglobulin (TG) is a homodimeric glycoprotein synthesized by the thyroid gland. To date, two hundred twenty-seven variations of the *TG* gene have been identified in humans. Thyroid dyshormonogenesis due to *TG* gene mutations have an estimated incidence of approximately 1 in 100,000 newborns. The clinical spectrum ranges from euthyroid to mild or severe hypothyroidism.

The purpose of the present study was to identify and characterize new variants in the *TG* gene. We report an Argentine patient with congenital hypothyroidism, enlarged thyroid gland and low levels of serum TG. Sequencing of DNA, expression of chimeric minigenes as well as bioinformatics analysis were performed.

DNA sequencing identified the presence of compound heterozygous mutations in the *TG* gene: the maternal mutation consists of a c.3001+5G>A, whereas the paternal mutation consists of p.Arg296*. Minigen analysis of the variant c.3001+5A performed in HeLa, CV1 and Hek293T cell lines, showed a total lack of transcript expression. So, in order to validate that the loss of expression was caused by such variation, site-directed mutagenesis was performed on the mutated clone, which previously had a pSPL3 vector change, to give rise to a wild-type clone c.3001+5G, endorsing that the mutation c.3001+5G>A is the cause of the total lack of expression.

In conclusion, we demonstrate that the c.3001+5G>A mutation causes a rare genotype, altering the splicing of the pre-mRNA. This work contributes to elucidating the molecular bases of TG defects associated with congenital hypothyroidism and expands our knowledge in relation to the pathologic roles of the position 5 in the donor splice site.

1. Introduction

Congenital hypothyroidism (CH) is a complex group of thyroid diseases which results from alteration in the biosynthesis of thyroid hormones (dyshormonogenesis, 15-20 % of cases) or in thyroid gland development (grouped under the name of thyroid dysembryogenesis or dysgenesis, 80-85 % of cases). Dyshormonogenesis has been associated to variants in the *SLC5A* [Spitzweg and Morris, 2010; Targovnik et al., 2017, 2020], *SLC26A4* [Bizhanova and Kopp, 2010; Wémeau and Kopp, 2017, Targovnik et al., 2020], *SLC26A7* [Bruellman et al., 2020b, Cangul, et al., 2018, Hermanns et al., 2020; Ishii et al., 2019; Zou et al., 2018], *thyroid peroxidase* (*TPO*) [Abramowicz et al., 1992; Ris-Stalpers and Bikker, 2010; Targovnik et al., 2017, 2020], *DUOX1* [Aycan et al., 2017; Bruellman et al., 2020b, Liu et al., 2019; Watanabe et al., 2019b; Zou et al., 2018], *DUOXA1* [Liu et al., 2019], *DUOX2* [Belforte et al., 2016; Grasberger, 2010; Moreno et al., 2002; Muzza et al., 2014; Muzza and Fugazzola, 2017; Park et al., 2016; Targovnik et al., 2020], *DUOXA2* [Muzza and Fugazzola, 2017], *iodotyrosine deiodinase* (*IYD*) [Moreno and Visser, 2010; Targovnik et al., 2017] and *thyroglobulin* (*TG*) [Citterio et al., 2019; Di Jeso and Arvan, 2016; Ieiri et al., 1991; Targovnik et al., 2010a, 2011, 2016, 2017, 2020] genes.

Thyroid dyshormonogenesis due to *TG* gene variants have an estimated incidence of approximately 1 in 100,000 newborns. The clinical spectrum ranges from euthyroid to mild or severe hypothyroidism. During the last decades, two hundred twenty-seven variants in the human *TG* gene have been reported associated with congenital goiter and also endemic and nonendemic goiter: 26 splice site variants (19 in the donor splice site and 7 in the acceptor splice site), 42 nonsense variants, 130 missense variants (18 located at in the wild type cysteine residues, 7 originating new cysteine residues, 27 in the ChEL-homology domain and 78 located along the remaining TG monomer), 5 duplications (4 singles and 1 multiple), 2 insertion (1 multiple and 1 involving a large number of nucleotides), 21 deletions (13 singles, 4 multiples and 4 involving a large number of nucleotides) and 1 imperfect DNA inversion [Abdul-Hassan et al., 2013; Agretti et

al., 2013; Alzahrani et al., 2006; Baryshev et al., 2004; Bruellman et al., 2020a, 2020b; Brust et al., 2011; Cangul et al., 2014; Caputo et al., 2007a, 2007b; Caron et al., 2003; Chen et al., 2018; Citterio et al., 2011, 2013a, 2013b, 2015; de Filippis et al, 2017; Fan et al., 2017; Fu et al., 2016a, 2016b; Gutnisky et al., 2004; Heo et al., 2019; Hermanns et al., 2013; Hishinuma et al., 1999, 2005, 2006; Hu et al., 2016; Ieiri et al., 1991; Jiang et al., 2016; Kahara et al., 2012; Kanou et al., 2007; Kim et al., 2008; Kitanaka et al., 2006; Liu et al., 2012; Lof et al., 2016; Long et al., 2018; Machiavelli et al., 2010; Makretskaya et al., 2018; Medeiros-Neto et al., 1996; Mendive et al., 2005; Mittal et al., 2016; Mizokami et al., 2019; Moya et al., 2011; Narumi et al., 2011; Nicholas et al., 2016; Niu et al., 2009; Pardo et al., 2008, 2009; Peteiro-Gonzalez et al., 2010; Raef et al., 2010; Rivolta et al., 2005; Rubio et al., 2008; Santos-Silva et al., 2019; Siffo et al., 2018; Sun et al., 2018; Tanaka et al., 2020; Targovnik et al., 1993, 1995, 2001, 2010b, 2012; van de Graaf et al., 1999; Wang et al., 2020; Watanabe et al., 2018, 2019, Wright et al., 2020; Yamaguchi et al., 2020; Yu et al., 2018; Zou et al., 2018; Corral et al., 1993; Pérez-Centeno et al., 1996; Gonzáles-Sarmiento et al., 2001]. The patients are typically homozygous or compound heterozygous for the gene mutations, and their parents are carriers of one of such variant.

In the present study we report a Argentinean patient with CH and low levels of serum TG. Screening by direct sequencing analysis of the *TG* gene revealed a previously reported, high-frequency nonsense *TG* mutation and a novel donor splice site (5'ss) *TG* mutation, constituting a compound heterozygous for c.886C>T [p.Arg296*] and c.3001+5G>A mutations. In our cellular expression system, the mutation at the 5'ss causes a total lack of expression of the transcript. Our results provide new insights on the role of the splice site mutations in the generation of CH and in particular about the effect of the c.3001+5G>A mutation on the *TG* splicing mechanism.

2. Materials and Methods

2.1 Patient

We report a boy born in 2010, is the third child of a non consanguineous couple. With a birth weight 3.075 g, he was born by cesarean section due to maternal cholestasis. Due to unremarkable history on delivery, he was exposed to iodinated desinfectants the first days of life. At 10 days of life he was referred to the Endocrinology Division of the Children's Hospital as a result of elevated TSH in neonatal screening at 48 hs of age (137 mU/L, cut off: 10). He appeared icteric, with a slightly prominent tongue and a palpated soft goiter. Serum thyroid profile showed elevated TSH of >100 mIU/L (normal range between 0.5 and 8) with low total T_4 of 3.7 µg/dl (normal range between 6 and 18), low free T_4 of 0.47 ng/dl (normal range between 1 and 2.6) and normal total T_3 of 103 ng/dl (normal range between 80 and 260). The serum TG concentration was 4 ng/ml (normal range between 30 and 100) suggesting that hypothyroidism could be related to defective TG synthesis. ^{99m}Technetium scintigraphy showed a hypercaptant goiter.

There was no historical evidence of iodine deficiency in the family, and the parents had no history of previous thyroid diseases.

He started treatment with levothyroxine with good adherence. Since then he grew and developed normally till age 6 when treatment was whithdrawn for a month and hypothyroidism was confirmed. A perchlorate discharge test was negative. Treatment was reintroduced.

Written informed consent to participate in the clinical and genetic studies was given by both parents and the research project was approved by the Institutional Review Board.

2.2 DNA sequencing

Genomic DNA was isolated from peripheral blood leucocytes by using standard methods. The 180 bp of the promotor region and all 48 exons of the *TG* gene, including splicing signals and the flanking intronic regions were amplified by PCR and 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 3500XL Genetic Analyzer (Applied Biosystems).

2.3 Construction and expression of the minigenes.

To study the effect of the c.3001+5G>A mutation, we constructed wild-type and mutated hybrid minigenes using the vector pSPL3 (Life Technologies Inc., Gaithersburg, MD). The genomic DNA region from index patient II-1 containing exon 11 (240-bp) and intronic flanking sequences (110-bp upstream from the 5' exon end and 367-bp downstream from the 3' exon end) were amplified by long PCR technique using elongase (Thermo Fisher Scientific, Waltham, MA). The forward primer (pSPL3I10NotI; 5'-**ATAAGAATGCGGCCGCGGTGTGTGTGGTGTGTAT**-3') contained the *Not*I site (underlined) and the reverse primer (pSPL3I11BamHI; 5'-**CAGGATCCTGTGTGGTGTTCCTGAATCC**-3') contained the *Bam*HI site (underlined). The 741-bp (717 of which were *TG* sequences) PCR products were purified from the agarose gel by use the GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK) and were sequentially digested with *Not*I and *Bam*HI. The insert was directionally cloned into the *Not*I and *Bam*HI sites of the pSPL3 vector. Because index patient II-1 is heterozygous for the c.3001+5G>A mutation, the wild-type and mutated alleles were thus cloned. The recombinant plasmids were amplified in DH5α-competent cells and purified by use of the QIAGEN Plasmid Plus Maxi Kit (QIAGEN, Venlo, Netherlands). The correct sequence was confirmed by sequencing with the intronic primer TG11F [Gutnisky et al., 2004].

HeLa, CV1 and Hek293T cell lines were grown in 3.8-cm dishes in DMEM supplemented with 10% bovine calf serum, in a 5% CO_2 atmosphere at 37 °C. When cells reached approximately 80% confluence, they were transfected with 500 ng plasmid DNA (wild-type, mutant, and control pSPL3)/3.8-cm dish with the Lipofectamine 2000 (Thermo Fisher Scientific). Twenty four hours

later, cells were harvested and total RNA was extracted with Trizol (Thermo Fisher Scientific). Reverse Transcription-PCR (RT-PCR) was performed as described previously [Gutnisky et al., 2004] using vector-specific primers: forward primer (pSPL3F), 5-tctgagtcacctggacaacc-3 and reverse primer (pSPL3R), 5-atctcagtggtatttgtgagc-3. Samples were heated to 94 °C for 5 min, followed by 40 cycles of DNA denaturation (94 °C for 30 sec), annealing (55 °C for 30 sec), and polymerization (72 °C for 1 min). After the last cycle, the samples were incubated for an additional 10 min at 72 °C. The identity of all the RT-PCR products was confirmed by cloning into pGEMT-Easy Vector System (Promega) and sequencing with the T7 primer.

2.4 Site-directed Mutagenesis

The pSPL3A-c.3001+5G M clone was generated from pSPL3A-c.3001+5A using Quick Change II Site Directed Mutagenesis Kit (Agilent, Santa Clara, CA) following the manufacturer's recomendations. Mutagenesis primers were designed using Quick Change Primer Design (http://www.genomics.agilent.com) forward primer: 5'-tggcggctcagtctagtgagtgtggtgccc-3', reverse primer: 5'-gggcaccacactcactagactgagccgcca-3'. The final construct was verified by sequencing with the intronic primer TG11F [Gutnisky et al., 2004].

2.5 Computer prediction analysis

Searching for potential 5'ss sequences in the *TG* gene spanning from exon 11 to intron 11 was accomplished using the NNSplice (http://www.fruitfly.org/seq_tools/splice.html), Fsplice (http://linux1.softberry.com/berry.phtml?topic=fsplice&group=programs&subgroup=gfind) and SPLM (http://linux1.softberry.com/berry.phtml?topic=splm&group=programs&subgroup=gfind) prediction tools. Scores of the 9-nt sequences, corresponding either to the authentic 5'ss and the mutated 5'ss, were calculated by means of the MaxEntScan program (http://genes.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html). Finally, the analysis of exon

sequences that allows the identification of putative exonic splicing enhancer (ESE) responsive to the human Ser/Arg-rich proteins (SR proteins) was performed using the ESEfinder program (http://rulai.cshl.edu/tools/ESE/).

2.6 Nucleotide and amino acid nomenclatures

The amino acid position is designated according to the reference sequences reported in NCBI accession number: NM_003235.5. The A of the ATG of the initiator methionine codon is denoted nucleotide +1. Amino acid positions are numbered including the 19-amino acids of the signal peptide.

3. Results

3.1 *DNA sequence analysis of TG gene.*

All 48 exons of the *TG* gene from index patient II-1 were screened as well as 180 bp of the *TG* promoter and all the flanking intronic sequences by direct DNA sequencing. Sequence analysis of 15,000 bases revealed that the index patient was heterozygous for a previously extensively documented nonsense mutation due to a cytosine to thymine transition at nucleotide 886 in exon 7 (c.886C>T, Chr 8:133894854C>T, dbSNP: rs121912648, ClinVar ID: 12695) (Figure 1a) which replaces the wild-type arginine at codon 296 with a stop codon [p.Arg296*]. The GT-AG splicing consensus sequences were rigorously preserved in all introns, except in the donor splice site of intron 11 where a heterozygous nucleotide substitution guanine to adenine was identified at position +5 (c.3001+5G>A, Chr 8:133906179G>A) (Figure 1a), compared with the expected sequence. This mutation was not reported in the literature or gnomAD database (https://gnomad.broadinstitute.org/).

Segregation analysis by direct sequencing of PCR products of exons 7 and 11 from the parents showed that the allele c.886C>T [p.Arg296*] was derived from her father and the allele c.3001+G>A from her mother (Figure 1a). This finding indicates that the index patient II-1 is a compound heterozygous for c.886C>T [p.Arg296*] and c.3001+5G>A mutations in the *TG* gene.

3.2 Minigene analysis

We tested the c.3001+5A mutation for abnormal splicing using the pSPL3 vector system (pSPL3B-c.3001+5A, Figure 1b and 2). Transfection with mutated pSPL3B-c3001+5A minigen, performed in HeLa, CV1 and Hek293T cell lines, showed a total lack of transcript expression, whereas wild type pSPL3A-c.3001+5G minigen (Figure 2) and pSPL3 control showed the expected fragments of 500 bp (α splicing event) and 260 bp (γ splicing event), respectively (Figure 3). A weak unpredicted band of approximately 380 bp was detected in mutated pSPL3B-c3001+5A

minigen (Figure 3). Sequence analysis of this cloned band showed that it contains a DNA fragment corresponding to the mitochondrial genome. This finding indicates that there is a partial homology of the primers used in RT-PCR (pSPL3F and pSPL3R) with the mitochondrial genome, originating a weak nonspecific band, especially when the target transcript is absent.

To examine whether the lack of expression in the mutated clone could be caused by a mutation in its vector pSPL3, the vector in the mutated clone was changed to the vector of the wild type clone. The insert from the recombinant pSPL3B-c.3001+5A clone, was introduced in the vector of the pSPL3A-c.3001+5G clone which previously showed expression of the expected transcript, giving rise to the new pSPL3A-c.3001+5A clone (Figure 2). However, the expression of the transcript remains null with the pSPL3A-c.3001+5A clone, despite the vector change (Figure 3). The weak nonspecific band of approximately 380 bp was also observed in the expression of this construct (Figure 3).

In order to validate that the loss of expression was caused by c.3001+5A variant, site-directed mutagenesis was performed on the pSPL3A-c.3001+5A mutated minigen to give rise to pSPL3A-c.3001+5G M wild-type clone (Figure 2), reestablishing the expression of wild type transcript (Figure 3), endorsing that the c.3001+5A variant is the cause of the total lack of expression.

3.3 *5' Splice site prediction analysis*

In order to evaluate in silico the relevance of the c.3001+5G>A mutation, wild-type and mutated 5'ss located in the intron 11 were analized using the NNSplice, FSplice and SPLM tools. As shown in Figure 4a, the wild-type 5′ss was recognized by all three programs, whereas the mutated 5′ ss did not identify as a donor site of splicing by NNSplice, FSplice and SPLM programs (Figure 4a). The strength of mutated and physiologic 5'ss sites were also compared by four other methods (MaxEntScan program), the maximum entropy model (MAXENT), the maximum dependence decomposition model (MDD), the first-order Markov model (MM) and Weight Matrix Model

(WMM), that require the prior knowledge of the input sequence to be tested. MAXENT, MDD, MM and WMM scores were consistently lower for the mutated 5′ ss with respect to wild type 5′ ss (Figure 4a).

The 5' ss consensus sequence is critical but often insufficient for the accurate 5'ss recognition and may require auxiliary sequences in both introns and exons. ESE elements are sequence tracts that are required for correct exon definition and inclusion [Blencowe, 2000]. ESE sequences act as binding sites for SR proteins (SF2/ASF, SC35, SRp40 and SRp55) [Blencowe, 2000]. ESE motifs were identified in the vicinity of the ss of constitutive and alternative exons [Wang et al., 2005]. Interestingly, according to ESEfinder, c.3001+5G>A mutation does not diminish the score or eliminates the SR proteins that bind to ESE sequences located around the exon/intron 11 junction (Figure 4b). Both mutated and wild type 5' ss were reconized by SF2/ASF, SC35, SRp40 and SRp55 proteins.

4.Discussion

We have studied an Argentinean patient that had clinical and biochemical criteria suggestive of CH associated with *TG* gene deficiency: lower serum TG and high levels of serum TSH with simultaneous low levels of circulating thyroid hormones [Targovnik et al, 2010a, 2011]. The low TG levels and a perchlorate discharge test negative are the basis for the selection of patients for molecular studies in the *TG* gene. Molecular analyses indicated that the affected individual carries a new compound heterozygous for p.Arg296* and c.3001+5G>A mutations in the *TG* gene.

p.Arg296* mutation (originally published as p.Arg277*) is the most frequently identified TG variant in Caucasian populations which has been found in families from Argentina, Brazil, Spain, Portugal, France, United Kingdom, Saudi Arabia and Iraq, as homozygous, compound heterozygous or monoallelic variants. [Abdul-Hassan et al., 2013; Brust et al., 2011; Caputo et al., 2007a, 2007b; Citterio et al., 2011, 2013a; Gutnisky et al., 2004; Machiavelli et al., 2010; Nicholas et al., 2016; Pardo et al., 2009; Peteiro-Gonzalez et al., 2010; Rivolta et al., 2005; Siffo et al., 2018; Santos-Silva et al., 2019; van de Graaf et al., 1999; Zou et al., 2018]. This mutation was available in heterozygous state from gnomAD database in Ashkenazi Jewish, Latino, Europeans (non Finnish), European (Finnish), African and South Asian populations with an estimated total Minor Allele Frequency (MAF) of 0.0003535 % (100/282,884) for the allele thymine[886].

The monomeric mature human TG [Malthiéry and Lissitzky, 1987; van de Graaf et al., 2001] is composed of a 19 amino acids signal peptide followed by a 2748 amino acid polypeptide. The classic model is organized in four structural regions (I, II, III and IV) [Citterio et al., 2019; Di Jeso and Arvan, 2016; Targovnik et al., 2016, 2017, 2020] (Figure 5a). Recently, Coscia et al., [2020] reported the 3D structure of human TG. This new model is organized in five structural regions: N-Terminal Domain (NTD), core, flap, arm and C-Terminal Domain (CTD) (Figure 5b). The region IV in the classical model or the CTD region in the new model are integrated by the cholinesterase-like (ChEL) domain (Figure 5a and 5b) [Park and Arvan, 2004; Swillens et al., 1986]. The most

important T_4-forming site couples donor DIT^{149} to acceptor DIT^{24} [Lamas et al., 1989; Palumbo et al., 1990; Dunn et al., 1998]; whereas the main T_3-forming site couples an MIT^{2747} at the antepenultimate residue of one TG monomer with the antepenultimate DIT^{2747} in the apposed monomer of the TG dimer [Citterio et al., 2018]. p. Arg296*variant comprises only a part of region I (classical model) or NTD region (new model) and eliminates the ChEL domain (Figure 5c) required for TG dimerization and functions as an intramolecular chaperone and as a molecular escort for the remaining regions [Lee et al., 2008]. The functional consequence of p.Arg296* truncated protein is the complete loss of the carboxy-terminal hormonogenic domains, and consequently, limited ability to generate thyroid hormone, particularly T_3 . However, p.Arg296* TG peptide retains its ability to synthesize T_4 because it still harbors both the acceptor Tyr^{24} and the donor Tyr^{149} (Figure 5c). It is possible to hypothesize that a short amino-terminal portion of TG with a single hormonogenic site even synthesizing low physiological levels of thyroid hormone was sufficient for support the vertebrate´s complexification from the first moments of its appearance until the ChEL domain fusion event. This hypothesis is supported by the observation that a milder hypothyroidism phenotype was described in some homozygous patients with p.Arg296* mutation [Caputo et al., 2007a; Citterio et al., 2013a; Pardo et al., 2009; Rivolta et al., 2005; Siffo et al., 2018; van de Graaf et al., 1999; Zou et al., 2018].

The second mutation identified in the index patient II-1 was a substitution of wild-type guanine for adenine at position +5 in the intron 11. Defects in pre-mRNA splicing represent one of the main causes of human genetic diseases, they vary according to each pathology between 10% and 50% [Roca et al., 2013], either by altering degenerate positions of donor or acceptor ss sequences or branch sites even by affecting intronic or exonic splicing regulatory sequences such as ESE elements [Bonnet et al., 2008; Tournier et al., 2008]. The human 5' consensus splice donor sequence is AG/GTRAGT (R indicates purine and backslashes indicate the exon-intron junction site) [Shapiro and Senapathy, 1987, Roca et al., 2003, Buratti et al., 2007]. At the 5'ss, mutations

affecting the guanine at position +1 are the most common, followed by mutations at position +5. The position +5guanine forms strong guanine-citosine base pairs with U1 small nuclear RNA (U1 snRNA) [Roca et al., 2013]. Mutations in this position significantly reduce the pairing of the 5' ss with the complementary sequence at the end of site in U1 snRNP [Roca et al., 2013]. The most common consequence of splicing mutations in higher eukaryotes is the skipping of one or more exons that precede it followed by creation or activation of cryptic donor and acceptor sequences (Roca et al., 2003; Buratti et al., 2007). In particular, a missplicing of *TG* pre-mRNA due to a mutation in consensus donor or acceptor ss is known to induce a congenital goiter and hypothyroidism in humans. Exon skipping in the human *TG* gene can be caused by nucleotide substitutions or deletion in acceptor or donor ss involving the -4/-3/-2/-1 (c.2762-4C>T, c.275-3C>G, c.5042-2A>G, c.6563-2A>G, c.2762-1G>A, c.6200-1G>C, c.7998-1G>A) or +1/+2/+3/+4/+5/+6 position (c.638+1G>A, c.745+1G>A, c.4816+1G>T, c.4932+1G>C, c.2176+1G>A, c.5686+1G>T, c.5686+1G>A, c.5686+1G>C, c.6262+1delG, c.6876+1delG, c.274+2T>G, c.7036+2T>A, c.7862+2T>A, c.5401+2T>C, c.4159+3_+4delAT, c.176+5G>T, c.638+5G>A, c.3001+6T>G, c.3433+3_+6delGAGT), respectively [Abdul-Hassan et al., 2013; Alzahrani et al., 2006; Bruellman et al., 2020b; Chen et al., 2018; Citterio et al., 2015, de Filippis et al., 2017; Fu et al., 2016a, Gutnisky et al., 2004; Hermanns et al., 2013, Hishinuma et al., 2006; Hu et al., 2016, Ieiri et al. 1991; Makretskaya et al., 2018; Medeiros-Neto et al., 1996; Narumi et al., 2011; Nicholas et al., 2016; Niu et al., 2009; Pardo et al., 2008, 2009; Peteiro-Gonzalez et al., 2010; Rubio et al., 2008; Targovnik et al., 1995, 2001, 2012; Watanabe et al., 2019; Zou et al., 2018]. Sato et al. (2014) reported in rodents a guanine to thymine transversion at the acceptor site of intron 6 of the *TG* gene (c.749-1G>T) which induced a complete missing of exon 7 from the *TG* transcript causing in homozygosity, dwarfism and goiter and in heterozygosity, only goiter. Three mutations affecting position 5 in the 5'ss have been found in the *TG* gene associated with congenital goiter and hypothyroidism, in introns 2 (c.176+5G>T; de Filippis et al., 2017), 5 (c.638+5G>A;

Nicholas et al., 2016) and 15 (c.3433+3_+6delGAGT; Nicholas et al., 2016). Moreover, twelve mutations at position +5 in introns of the *TG* gene were communicated by gnomAD database (c.638+5G>A, intron 5; c.3139+5G>A, intron 12; c.4002+5A>G and 4002+5A>C, intron 18; c.5233+5T>A and c.5233+5T>C, intron 26; c.6262+5G>A, intron 35; c.6397+5G>A, intron 36; c.7404+5G>A, intron 42, c.7572+5G>A, intron 43; c.7862+5G>A and 7862+5G>C, intron 45).

The usefulness of splicing reporter minigene assays has been shown to be a good approach to determine the effect of the variants on the splicing process [Bonnet et al., 2008; Tournier et al., 2008] when is difficult to obtain RNA from patients' tissues. A high level of concordance between data obtained with these assays and data from patient's RNA has been shown [Bonnet et al., 2008]. Since the thyroid tissue and blood cells from II-1 are unavailable we used hybrid minigen constructs to evaluate the c.3001+5G>A mutation. In the present study, minigenes were constructed using the pSPL3 vector, which has a minimal gene organization: the SV40 promoter followed by an exon-intron-exon structure with a multiple cloning site located inside the intron. These constructions are expressed in living cells where the splicing machinery remains intact. Surprisingly, the minigen analysis of the c.3001+5A variant shows a total lack of transcript expression. Interestingly, the restoring the wild-type minigene from the mutated clone by site-directed mutagenesis confirms that the mutation c.3001+5G>A is responsible for the total collapse of the expression of the transcript, in our cellular expression system. Previous report showed that a mutation at donor site in the splice region +5 (c.1249+5G>A) in the *SERPING1* gene produces a complete degradation of the mutant allele mRNA in a case of familial hereditary angioedema [Colobran et al., 2014]. Roest et al (1996) identified another example of mutation inactivating in position +5, a substitution of guanine to cytosine at intron 64 of the dystrophin gene, disrupting the splice donor consensus sequence and activating a cryptic splice donor site 57 bp downstream (Roest et al., 1996). These observations remark and expand the importance of the finding and characterization of our variant c.3001+5A since similar pre-RNA events caused by mutations in +5adenine region seems to be responsible for

phatologies also in other genes. One possible explanation for missing of transcript is that the mutation causes instability of the mRNA, making it a potential target for degradation. Nonsense-mediated mRNA decay (NMD) is one type of mRNA surveillance mechanism which ensures the rapid degradation in the cytoplasm of transcripts containing nonsense codons, thereby preventing the accumulation of truncated and potentially harmful proteins [Behm-Ansmant et al. [2007]. However, we cannot properly say that NMD is the mechanism responsible for the complete loss of the transcript in our cellular expression system. The pSPL3 expression vector methodology was used with the purposes of studying signaling of spliceosome through the generation of transcripts. The pSPL3 system is not suitable for a correct reading frame analysis. On the other hand, in the patient II-I, since the exon 11 skipping conserves the reading frame, to generate a truncated protein, which activates NMD machinery as it was observed in the *SERPING1* gene [Colobran et al., 2014], the c.3001+5G>A mutation must activate a exonic or intronic cryptic ss. We want to point out that another posible reason is that the c.3001+5A variant affects early stages of the transcription, which could explain the total absence of transcripts from the mutated allele. In addition to NMD there are RNA surveillance mechanisms that act in the nucleus while transcripts are associated with the chromatin and contribute to down-regulate the expression of abnormal mRNAs.

Vas-Drago et al. [2015] detected decreased transcription of the *MARVELD2 3'SM* gene, which contains a 3' ss mutation in the third intron. This observation raises the possibility that additional mechanisms are involved in coupling transcription to splicing efficiency. Unfortunately, we did not have thyroid tissue or fresh peripheral blood from the patient II-1 to confirm the total lack of the mutated allele *in vivo*.

Four different conclusions emerged from our study. First, the sequencing of the human *TG* gene revealed that a new compound heterozygous mutations, p.Arg296*/c.3001+5G>A causes the CH phenotype in the index II-1 patient. Second, minigen analysis of the variant c.3001+5A shows a total lack of transcript in our cellular expression system. Third, bioinformatic performance shows

that such mutation does not diminish the score or eliminates the SR proteins that bind to ESE sequences located around the exon/intron 11 junction, Finally, we propose two hypotheses about such interesting and unusual new variant c.3001+5A: a complete degradation of the transcript from the mutated allele or that such variant could produce a total collapse of transcription in early regulatory stages. These studies confirm the allelic heterogeneity of the *TG* gene mutations; such feature makes it a perfect model for expanding our knowledge in relation to the genetics molecular mechanisms which still remain without more clear explanations, making of crucial clinical importance characterizing the molecular processes involved in the CH phenotype by TG defects.

Figure legends

Figure 1. ***Mutations in the thyroglobulin gene in index patient II-1 and their parents. a*****)** Family pedigree and mutations. The pedigree shows the pattern of inheritance of the mutant *thyroglobulin* alleles. Squares represent males and circles females. Filled symbols denote affected individuals and half-filled symbols, unaffected heterozygote individuals. The hatched symbols indicate the c.886C>T [p.Arg296*] mutated allele and the solid symbols the c.3001+5G>A mutated allele. Partial sequencing chromatograms of genomic DNA are shown (sense strand). Arrows denote the position of identified mutations, single chromatogram peaks indicate homozygosity and two overlapping peaks at the same locus, heterozygosity. Note that the index patient II-1 have inherited one copy of the c.886C>T [p.Arg296*] mutation from their father (I-1) and one copy of the c.3001+5G>A mutation from their mother (I-2). **b)** Genomic nucleotide sequence of intron 10/exon 11/intron 11 boundaries and their flanking intronic regions from index patient II-1, amplified by long PCR technique and cloned into the pSPL3 vector. The position of the oligonucleotide primers for amplified and cloning are underlined. Arrow denotes the position of identified mutation.

Figure 2. Minigen constructs strategy. The 741-bp PCR amplified fragments from index patient II-1 were directionally cloned into the NotI and BamHI sites of the pSPL3 vector. The resulting clones pSPL3A-c.3001+5G (wild-type) and pSPL3B-c.3001+5A (mutated) were expressed in eukaryotic cells. To rule out that the lack of expression in the pSPL3B-c.3001+5A mutated clone could be caused by a mutation in its vector pSPL3, the vector in the mutated clone was changed to the pSPL3 vector of the pSPL3A-c.3001+5G wild-type clone, originating pSPL3A-c.3001+5A mutated clone. Finally, in order to validate that the loss of expression was caused by c.3001+5A variant, site-directed mutagenesis was performed on the pSPL3A-c.3001+5A mutated minigen to give rise to pSPL3A-c.3001+5G M wild-type clone. Sense strand is shown. Arrows in the partial sequencing chromatograms denote the position of identified mutations.

Figure 3. ***Cellular expression of the wild-type and mutant minigenes performed in HeLa cell lines.*** Schematic representation of the genomic organization of the wild-type and empty pSPL3 vector minigenes and their RT-PCR products, α and γ splicing events, respectively. Vector and genomic DNA splice donor (GT) and acceptor (AG) sites are shown. cDNA was synthesized from transcribed mRNA and amplified with pSPL3F and pSPL3R primers complementary to flanking vector sequences. Gel electrophoresis of the RT-PCR amplification products (pSPL3B-c.3001+5A, pSPL3A-c.3001+5A, pSPL3A-c.3001+5G M, pSPL3A-c.3001+5G, empty pSPL3A vector, untransfected and negative RT-PCR control without RNA) and partial sequencing chromatograms (sense strand) corresponding to α and γ splicing events are shown. Transfection with mutated pSPL3B-c.3001+5A and pSPL3A-c.3001+5A minigenes showed a total lack of transcript expression, whereas pSPL3A-c.3001+5G M and wild type pSPL3A-c.3001+5G minigenes showed the expected fragments of 500 bp and pSPL3 control showed the expected fragments of 260 bp. White arrow denote the weak nonspecific band that contains a mitochondrial genome DNA fragment. The size marker is a 100 bp ladder (M). For interpretation of the minigene constructs see Figure 2.

Figure 4. ***c.3001+5G>A mutation, in silico analysis of the wild-type and mutated 5′ splice sites.*** **a)** Individual scores for each 5′ splice sites obtained by a panel of donor site prediction programs (NNSplice, FSplice, SPLM, MAXENT, MDD, MM and WMM). **b)** Potential SR binding sites (SF2/ASF, SC35, SRp40 and SRp55) identified by the ESEfinder 3.0 program. The height of each bar represents the motif scores, whereas its width indicates the length of the binding site motifs for SR proteins and its position along the sequence. Note that the wil-type and mutated 5′ splice site are potentially recognizable by SR proteins. / denotes the exon/intron boundary.

Figure 5

Structural organization of the wild-type and p.Arg296* mutant thyroglobulin proteins. **a)** Classical primary structure of human thyroglobulin. Schematic representation adapted from Holzer et al. [2016]; Malthiery and Lissitzky [1987]; Mercken et al. [1985]; Molina et al. [1996], Parma et al. [1987], Swillens et al. [1986] and van de Graaf et al. [2001] is showed. The thyroglobulin signal peptide (SP), repetitive units of TG type 1, TG type 2 and TG type 3, linker and hinge domains and the acetylcholinesterase-homology (ChEL) domain, drawn to scale, are represented by boxes. Thyroglobulin monomer is organized in four structural regions (I, II, III and IV). N-terminal T_4 (coupling of a donor DIT^{149} with the acceptor DIT^{24}) and C-terminal T_3 (coupling of a MIT^{2766} at the antepenultimate residue of one TG monomer with the antepenultimate DIT^{2766} in the opposite monomer) forming sites are shown**. b)** New model of thyroglobulin primary structure. Schematic representation adapted from Coscia et al. [2020]. The thyroglobulin signal peptide (SP), repetitive units of TG type 1 and TG type 3, and similar TG type 1, helical, Ig-like 1, Ig-like 2 and TNF/EGF/laminin-like fold domains, and the acetylcholinesterase-homology (ChEL) domain, are drawn to scale and represented by boxes. Thyroglobulin monomer is organized in five structural regions: N-Terminal Domain (NTD), Core, Flap, Arm and C-Terminal Domain (CTD). **c)** Primary structure of putative p.Arg296* mutant thyroglobulin protein.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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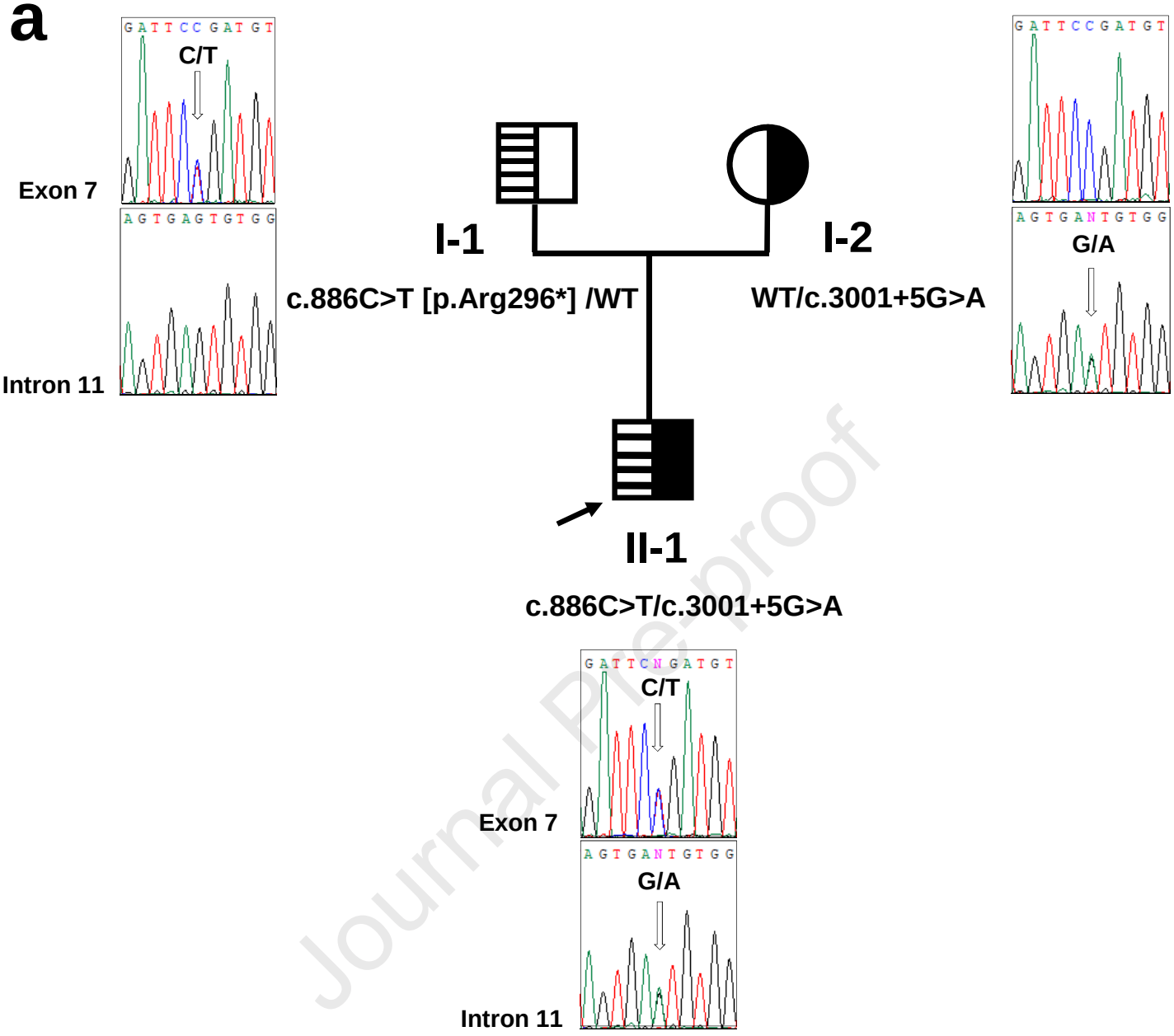

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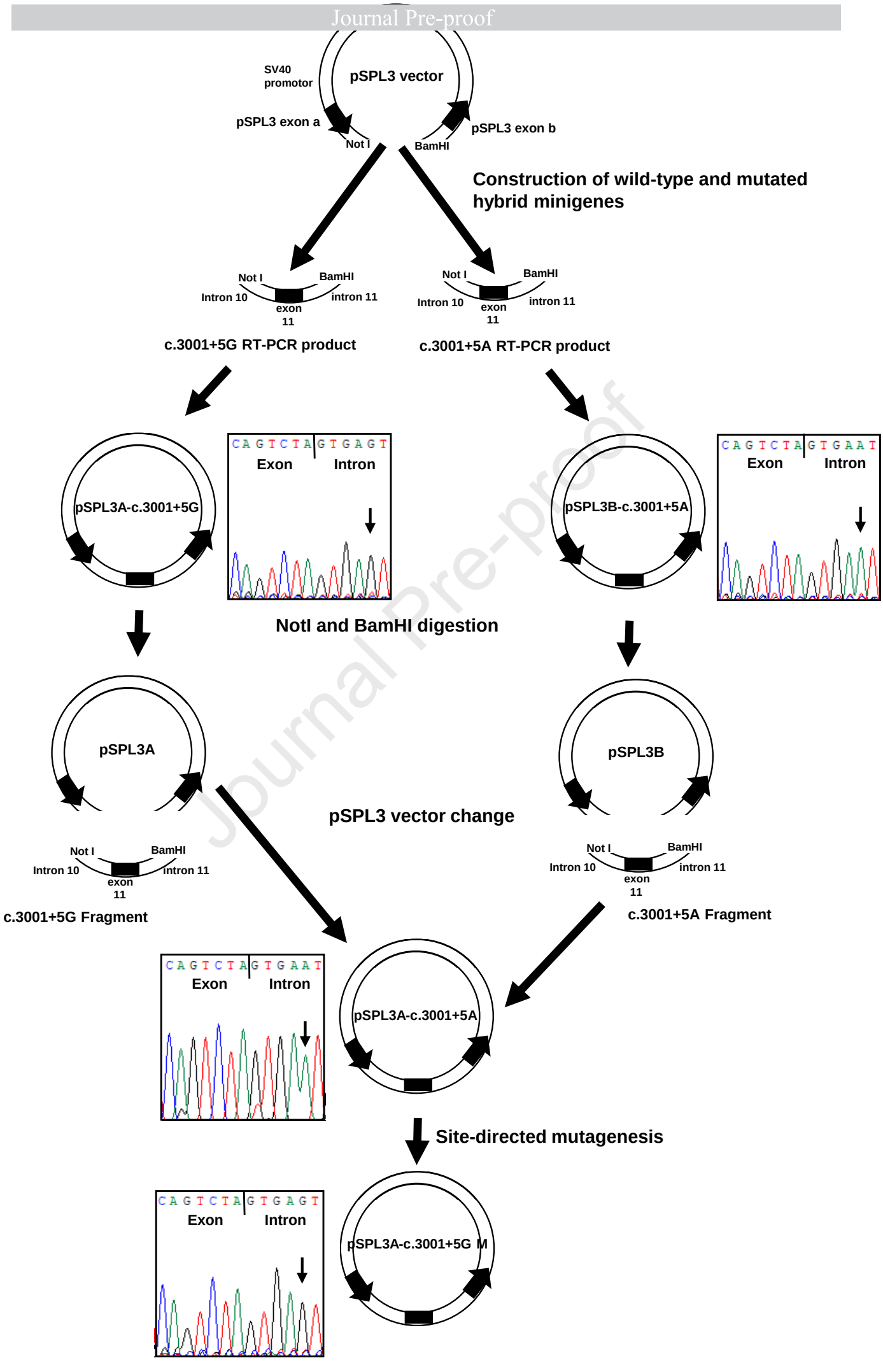
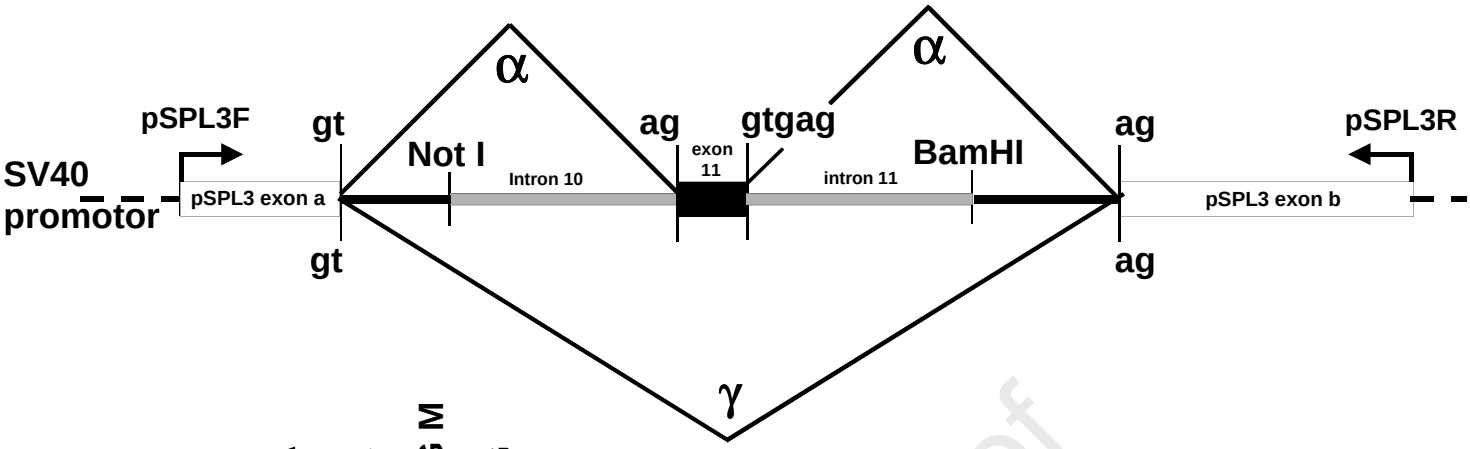
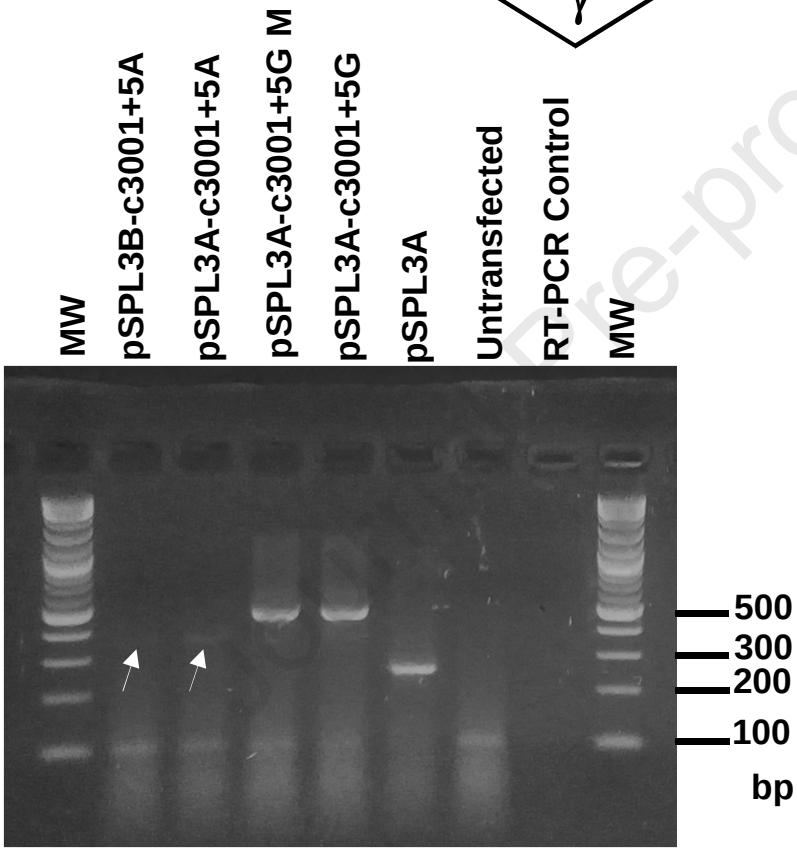
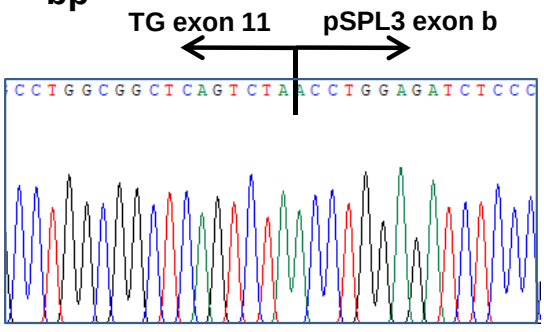
Intron 11



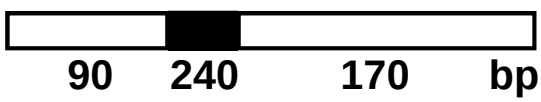


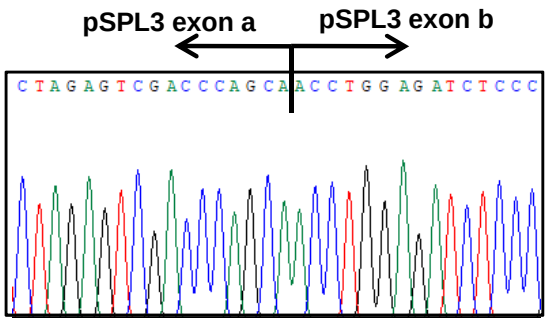




pSPL3A-c.3001+5G RT-PCR product

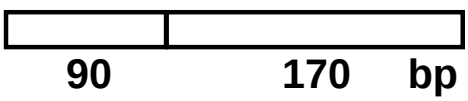
α: 500 pb





pSPL3A RT-PCR product

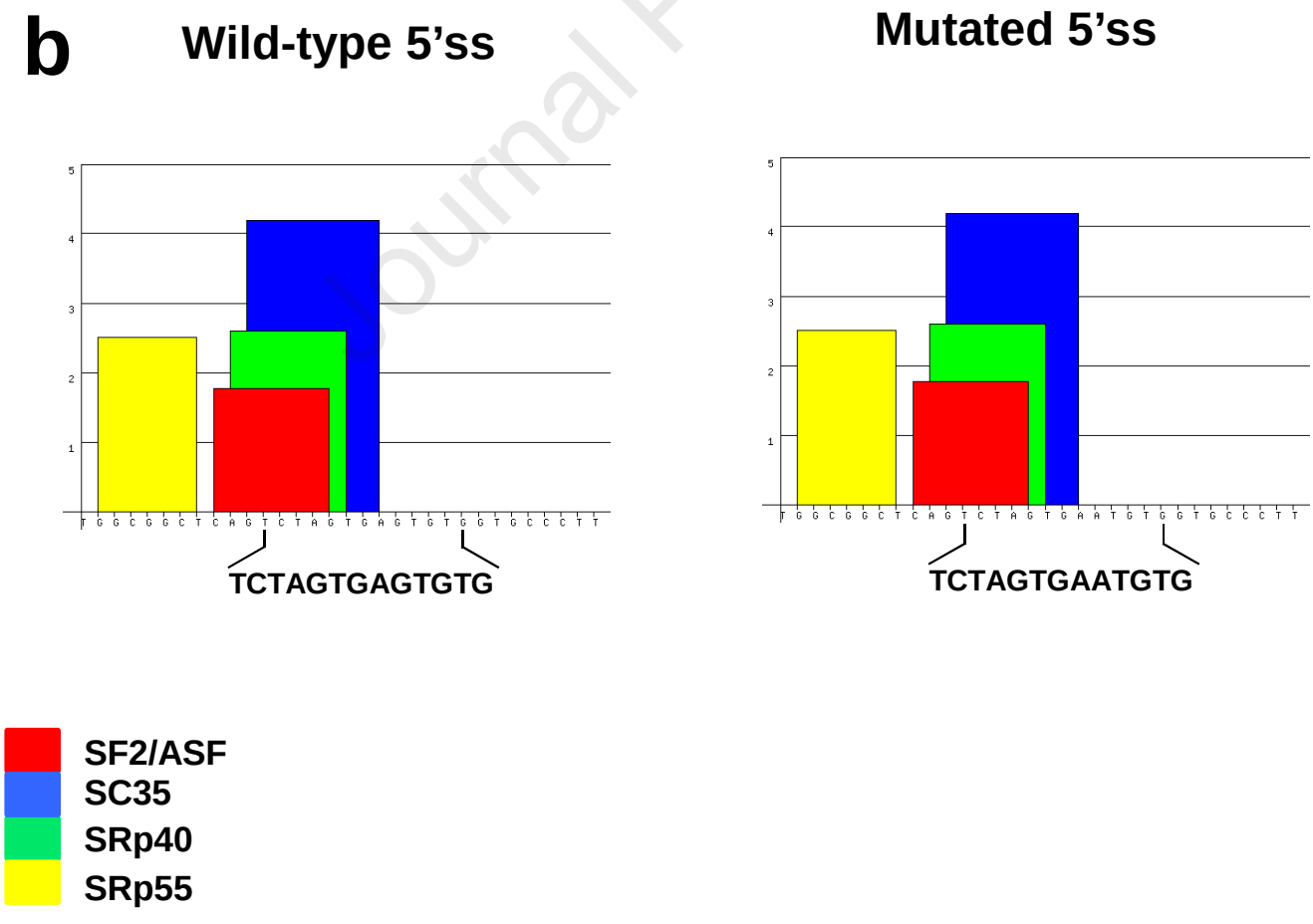
γ: 260 pb



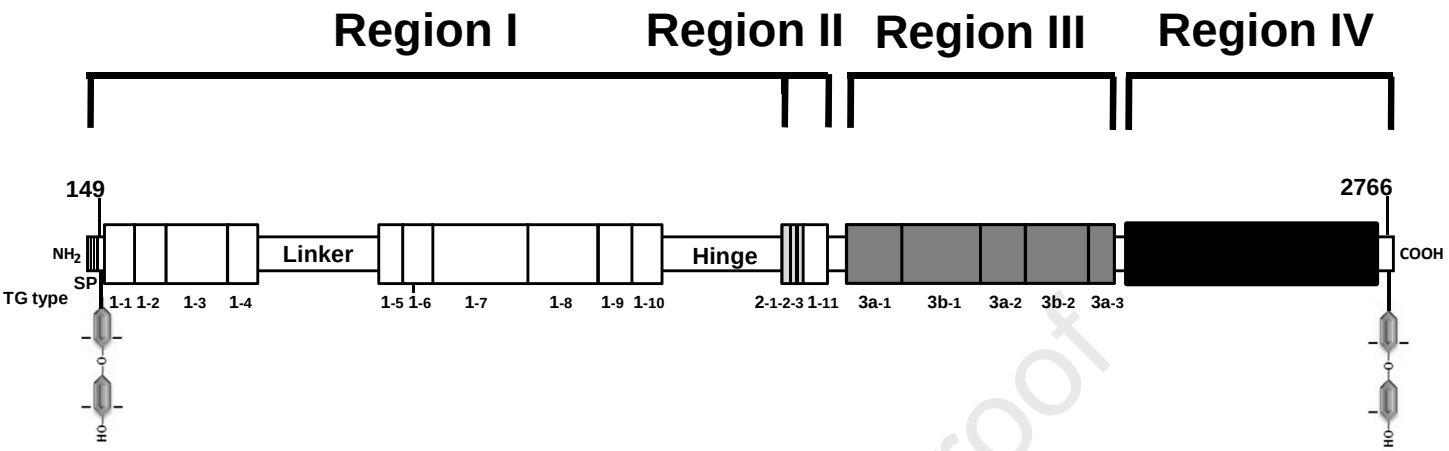
a

	Wild-type 5'ss	Mutated 5'ss
	CTA/GTGAGT	CTA/GTGAAT
NNsplice	0.92	0
FSplice	8.90	0
SPLM	29	0
MAXENT	7.43	-2.68
MDD	11.48	0.68
MM	5.90	1.43
WMM	6.49	3.04

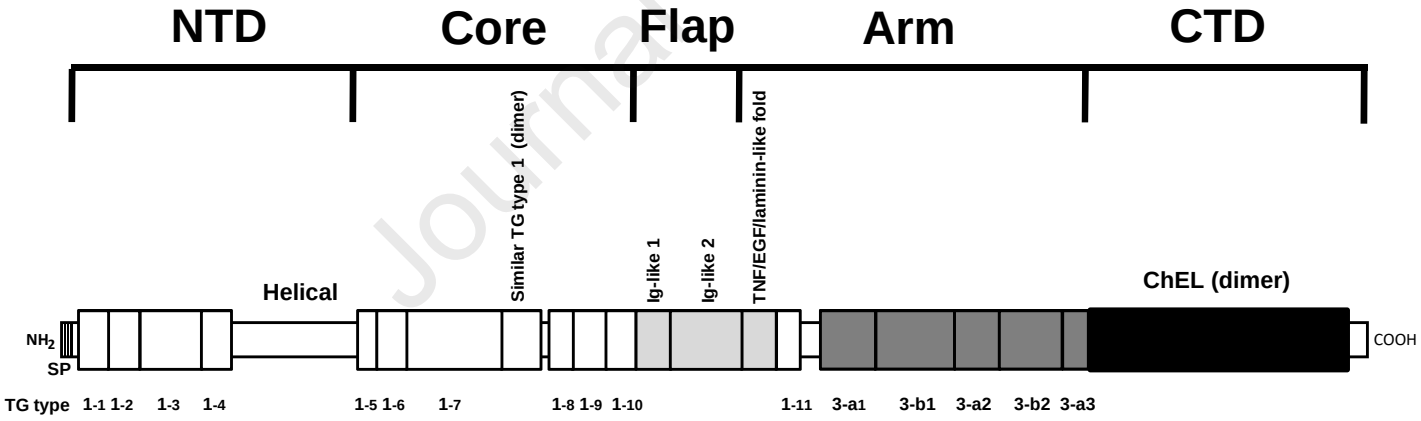
b



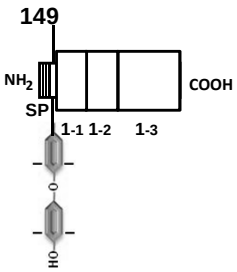
a) Classical model of thyroglobulin primary structure



b) New model of thyroglobulin primary structure



c) p.Arg296*



Highlights

- We report a patient with hypothyroidism by mutations in the thyroglobulin gene
- Molecular analysis revealed a compound heterozygous for p.R296*/c.3001+5G>A mutations.
- Minigen analysis shows a total lack of transcript expression.
- We demonstrate that the c.3001+5G>A mutation alters the splicing of the pre-mRNA.
- Our results confirm the genetic heterogeneity of thyroglobulin defects.