Journal Pre-proof

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PII: S0303-7207(23)00099-0

DOI: https://doi.org/10.1016/j.mce.2023.111948

Reference: MCE 111948

To appear in: *Molecular and Cellular Endocrinology*

Received Date: 20 March 2023

Revised Date: 7 May 2023

Accepted Date: 8 May 2023

Please cite this article as: Gomes Pio, M., Adrover, E., Miras, M.B., Sobrero, G., Molina, M.F., Scheps, K.G., Rivolta, C.M., Targovnik, Hé.M., The p.Cys1281Tyr variant in the hinge module/flap region of thyroglobulin causes intracellular transport disorder and congenital hypothyroidism, *Molecular and Cellular Endocrinology* (2023), doi: https://doi.org/10.1016/j.mce.2023.111948.

Credit Author Statement

M.G.P. contributed to Sanger sequencing, performing structural modeling analysis, site-directed mutagenesis, transfection, and TG protein secretion analysis. E.A. contributed to TG protein secretion analysis. M.B.M. and G.S. were involved in the recruitment of patients and acquisition of clinical data and blood samples. M.F.M. and K.G.S. contributed to bioinformatic analysis. C.M.R. contributed to the acquisition of funds and the design of the study. HMT contributed to bioinformatics predictive analysis, funding acquisition, study conception and design, and writing of the article. All authors critically reviewed and participated in manuscript revision and approved the final draft.

The p.Cys1281Tyr variant in the hinge module/flap region of thyroglobulin causes intracellular transport disorder and congenital hypothyroidism.

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Short title: p.Cys1281Tyr Thyroglobulin Gene Variant

Key words: Congenital Hypothyroidism, Thyroglobulin gene, p.Cys1281Tyr, Hinge module/flap region, Intracellular retention.

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Abstract

Congenital hypothyroidism (CH) due to thyroglobulin (TG) variants causes very low serum TG levels with normal or enlarged thyroid glands, depending on the severity of the defect, and with autosomal recessive inheritance. The purpose of this study was to functionally characterize p.Cys1281Tyr variant in the TG gene in order to increase our knowledge of the molecular mechanisms associated with CH. In order to find evidence that support the hypothesis that the p.Cys1281Tyr variant would affect the TG folding were performed amino acid prediction, 3D modeling and transient expression analysis in HEK293T cells. 18 of the 21*"in silico"* algorithms predict a deleterious effect of the p.Cys1281Tyr variant. The full-length 3D model p.Cys1281Tyr TG showed disulfide bond cleavage between the cysteines at positions 1249 and 1281 and rearrangement of the TG structure, while transient expression analysis indicated that p.Cys1281Tyr causes retention of the protein inside the cell. Consequently, these results show that this pathogenic variant makes it impossible for TG to fulfill its function in the biosynthesis process of thyroid hormones, causing CH. In conclusion, our results confirm the pathophysiological importance of misfolding of TG as a consequence of p.Cys1281Tyr variant located in the hinge module/flap region of TG.

Introduction

Congenital hypothyroidism (CH) is a deficiency of thyroid hormone (TH) presente from birth, affected about 1 in 1,500 to 1 in 4,000 newborns, and is characterized by elevated levels of thyroid-stimulating hormone (TSH) and low serum TH levels [Jing and Zhang, 2022, Kwak, 2018; Moran et al., 2022; Stoupa et al., 2021; Van Trotsenburg et al., 2021]. Children born with severe TH deficiency can develop intellectual disability if the condition is not treated early. Primary congenital hypothyroidism is classified into two major groups, thyroid dysgenesis and thyroid dyshormonogenesis (TDH) [Jing and Zhang, 2022, Kwak, 2018; Moran et al., 2022; Van Trotsenburg et al., 2021]. The group of thyroid dysgenesis, includes thyroid glands that are absent (agenesia or athyreosis), reduced in size (hypoplasia) or located in an unusual position (thyroid ectopia) [Jing and Zhang, 2022, Kwak, 2018; Moran et al., 2022; Van Trotsenburg et al., 2021]. Variants in *NKX2.1*, *NKX2.5, FOXE1, PAX-8, TSHR, CDCA8, ELN, GLIS3, HOXD3, HOXB3, JAG1, KMT2D, TBX1*, *TUBB1*, and URBI genes were identified in patients with thyroid dysgenesis phenotype [Kwak, 2018; Larrivée-Vanier et al., 2022; Zou et al., 2018]. TDH is a genetic defect that affects TH production due to deleterious variants in genes that code for proteins involved in the multiple steps of TH biosynthesis: *Solute Carrier Family 5 Member 5* (*SLC5A5, encoding NIS*), *Solute Carrier Family 26 Member 4* (*SLC26A4, encoding pendrin*), *Solute Carrier Family 26 Member 7* (*SLC26A7*), *Thyroid Peroxidase* (*TPO*), *Dual Oxidase 1* (*DUOX1*), *DUOX Maturation Factor 1* (*DUOXA1*), *Dual Oxidase 2* (*DUOX2*), *DUOX Maturation Factor 2* (*DUOXA2*), *Iodotyrosine Deiodinase* (*IYD*) *and Thyroglobulin* (*TG*) [Jing and Zhang, 2022, Kwak, 2018; Moran et al., 2022; Van Trotsenburg et al., 2021]. Also, variants in the *SLC26A4*, *SLC5A5*, *TPO*, *DUOX1*, *DUOX2* and *TG* genes were associated with thyroid dysgenesis [de Filippis et al., 2017; Kizys et al., 2017; Larrivée-Vanier et al., 2022; Makretskaya et al., 2018; Sun et al., 2018, Wang et al., 2020]. Patients with TDH due to *TG* gene variants have an autosomal recessive pattern of inheritance and a clinical range from

euthyroidism to permanent CH, with goiter. The incidence is estimated at 1 in 67,000 to 1 in 100,000 live births [Hishinuma et al., 2006; Van Graaf et al., 1999].

TG is a complex dimeric glycoprotein secreted by the thyroid cell at the apical membrane-colloid interface. The amino-terminal and central region of the monomeric TG preprotein include three types of repetitive motifs (TG type 1, TG type 2, and TG type 3) whereas the amino-terminal region is integrated by the cholinesterase-like (ChEL) domain. [Holzer et al., 2016; Malthiéry & Lissitzky, 1987; Mercken et al., 1985; Molina et al., 1996, Parma et al., 1987; van de Graaf et al., 2001]. Recently, the 3-dimensional atomic structure of human an bovine TG has been reported [Adaixo et al., 2022; Coscia et al., 2020; Kim et al., 2021; Marechal et al., 2022]. More than two hundred and ninety variants deleterious variants in the human *TG* gene have been reported associated to TDH (missense variants in the wild type cysteine residues and in the ChEL-homology domain, singles and multiple duplications, single and multiple deletions, multiple insertion, imperfect DNA inversion, aceptor and donor splice site variants and nonsense variants) [Citterio et al., 2021; Pio et al., 2021].

In the present study we report that the human p.Cys1281Tyr TG variant, in the hinge module/flap region, causes significant intracellular retention in HEK293T cells and further confirm that the cysteines and their integration into intrachain disulfide bonds plays a key role in the intracellular trafficking of the TG.

Materials and Methods

In order to find evidence that support the hypothesis that the p.Cys1281Tyr variant would affect the TG folding were performed amino acid prediction, 3D modeling and transient expression analysis.

Amino acid prediction analysis

p.Cys1281Tyr variant was analyzed with the sequence based predictors included in the VarSome tool [https://varsome.com]: REVEL, DEOGEN2, EIGEN, EIGEN PC, FATHMM, FATHMM-MKL, FATHMM-XF, LIST-S2, MudPred, LRT, M-CAP, MVP, Mutation assessor, Mutation Taster, PROVEAN, Polyphen2 HDIV, Polyphen2 HVAR, PrimateAI, SIFT, SIFT4G, EVE.

3D modeling analysis

The UCSF Chimera program was used to obtain the 3D model of the human and bovine TG (Pettersen et al., 2004; UCSF Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, https://www.cgl.ucsf.edu/chimera/)**.**

Site-directed Mutagenesis

The 8,304-bp full-length cDNA of TG rat was previously directionally cloned into the NotI and XbaI sites of the pcDNA6/V5-His B expression vector (Thermo Fisher Scientific), which we called the prTGwt clone [Citterio et al., 2020]. The 73% homology between rat and human TG (Supplementary Figure 1) makes the use of prTGwt of great consistent and representative value as previously shown [Citterio et al., 2020; Siffo et al., 2023]. The prTG[p.Cys1281Tyr] mutated clone was generated from prTGwt using QuikChange Lightning 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 (3842G>AF): 5'-cactacccagagcct**A**tcagaggcctcag-3', reverse primer (3842G>AR): 5'-

ctgaggcctctga**T**aggctctgggtagtg-3'. The final construct was verified by sequencing with the primers listed in Citterio et al. [2020], with the Big Dyedeoxyterminator Cycle Sequencing Kit (Applied Biosystems, Weiterstadt, Germany). The samples were analyzed on the 3500XL Genetic Analyzer (Applied Biosystems).

Cell Culture and Transfection

HEK293T cells obtained from ATCC were grown in a 6-plate well in Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific, Waltham, MA) containing 10% Fetal Bovine Serum (FBS, Thermo Fisher Scientific), penicillin (100 units/ml, Thermo Fisher Scientific) and streptomycin (100 µg/ml, Thermo Fisher Scientific) (designated as complete medium) at 37°C in a humidified 5% CO_2 incubator to approximately 70–80% confluence. Then, 0.5 µg of plasmid DNA were transiently transfected using Escort™ IV Transfection reagent (Sigma-Aldrich, Saint Louis, MO) in Opti-MEN medium (Thermo Fisher Scientific), according to the manufacturer's instructions. 6 h post-transfection, Opti-MEN medium was replaced by fresh complete medium in each well. Because serum TG contribute to the background in the Western blotting experiments [Park, Y.-n., Arvan, P., 2004], after 24 h of incubation, the complete medium was replaced by fresh complete medium without FBS and the cultures were incubated for an additional 20 h.

TG protein secretion analysis

Supernatant from the transfected cultures underwent two cycles of clarification at 4°C (5 min at 800 x g followed by 10 min at 10,000 x g) to remove cellular debris. The clarified supernatants were then diluted with 4X Laemmli buffer and heated at 95°C for 3 min. The cells were directly lysed in 4X Laemmli buffer and heated at 95°C for 3 min. Supernatants and cell lysates were then resolved in 7 % SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes. Primary rabbit monoclonal anti-TG antibody (ab156008, Abcam, Cambridge, UK) was diluted 1:3000 in PBS-

0.05 % Tween and incubated over night at 4 °C. HRP-conjugated goat anti-rabbit IgG antibody (ADI-SAB-300, Enzo Life Sciences Ltd, Exeter, UK) was diluted 1:6000 in PBS-0.05 % Tween and incubated for 2 h at room temperature. Bands were visualized using the Bio Lumina kit as directed by the manufacturer (Kalium Technologies, Quílmes, Argentina). Images were captured in a C-Digit Blot Scanner via Image Studio Software (LI-COR, Lincoln, NE).

Results

Amino acid prediction analysis

The *"in silico"* algorithms REVEL, DEOGEN2, EIGEN, EIGEN PC, FATHMM, FATHMM-MKL, FATHMM-XF, LIST-S2, MudPret, LRT, M-CAP, MVP, Mutation assessor, Mutation Taster, PROVEAN, Polyphen2 HDIV, Polyphen2 HVAR, PrimateAI, SIFT, SIFT4G, EVE were used to predict the pathogenicity levels of p.Cys1281Tyr. 18 of the 21 programs predict a deleterious effect of the p.Cys1281Tyr variant, only FATHMM, LIST-S2 and PrimateAI predict a tolerated effect.

3D modeling analysis of the identified p.Cys1281Tyr mutant

A cryo-electron microscopy structure of human TG (PDB 6SCJ, resolution: 3.60 Å, Coscia et al., 2020) was used to generate a full length mature TG structure model. In Figure 1A the wild-type human TG monomer is shown with its four regions according to the classical model. We can see that the affected amino acid cysteine^{1281} is located in an inner area of the protein, near to the TG region II and 16 amino acids upstream of the extensively analyzed p.Cys1264Arg pathogenic variant [Baryshev et al., 2004; Heo et al., 2019; Hishinuma et al., 1999, 2005, 2006; Kanou et al., 2007; Narumi et al., 2011; Wright et al., 2021; Yoon et al., 2020] present in the same hinge module of region I (Figure 1B).

For the structural analysis, bovine TG PDB 7N4Y [Kim et al., 2021] was used due to its better resolution, 2.61 Å, in contrast to the human TG PDB 6SCJ, in order not to lose details and analyze the elements involved in the analyzed area. The primary amino acid sequence of bovine TG PDB 7N4Y does not match the canonical bovine sequence entry in UniProt P01267. Note that the cysteine^{1249} and cysteine^{1281} residues in canonical human UniProt P01266 corresponds to cysteine^{1249} and cysteine^{1281} in bovine UniProt P01267, respectively, and to the cysteine^{1250} and cysteine^{1282} in bovine TG PDB 7N4Y and bovine UniProt A0A4W2CHS8, respectively.

In Figure 2A, the wild-type bovine TG cysteine[1282] (cysteine[1281] in humans) is seen surrounded by nine hydrogen bonds and forming a disulfide bond with wild-type bovine TG cysteine[1250] (cysteine[1249] in humans). When the bovine p.Cys1282Tyr (p.Cys1281Tyr in humans) change was made, the disulfide bond cleavage between both cysteines is clearly observed, apparently without interfering with any of these nine hydrogen bonds (Figure 2B). The bovine p.Cys1282Tyr (p.Cys1281Tyr in humans) variant shows the appearance of clashes (Figure 2C), suggesting that bovine tyrosine[1282] (tyrosine[1281] in humans) generates a structural rearrangement between the affected amino acid and the surrounding residues, where hydrogen bonds 4, 5, 6, 7, 8 and 9 could be affected.

Transient expression of p.Cys1281Tyr full-length rat thyroglobulin cDNA

As a last stage in the analysis of the p.Cys1281Tyr sequence variant (also p.Cys1281Tyr in the rat TG), functional assays were carried out to assess whether or not the presence of this change could be affecting the intracellular transport of TG and, consequently, its correct functionality. The prTG[p.Cys1281Tyr] mutated clone generated (Figure 3A) was transfected in HEK293T cells and the cell lysate and the extracellular supernatant proteins were analysed by Western blot using an anti-TG antibody. As observed in Figure 3B, the TG generated by prTG[p.Cys1281Tyr] mutated clone is retained inside the cell (complete lack of TG in the supernatant) while the TG from prTGWT wild-type clone is found both inside the cell and in the extracellular medium. These results would be indicating that the p.Cys1281Tyr variant in the *TG* gene would be the cause of the retention of the protein inside the cell, preventing its transport to the extracellular medium. If we extrapolate these results to patients who have this variant, we could say that it prevents the transport of TG towards the thyroid follicular lumen, making it impossible for it to fulfill its function in the biosynthesis process of thyroid hormones and thus causing a thyroid disease.

Discussion

In the present study we report a detailed study of the human p.Cys1281Tyr *TG* variant. The main purpose was to analyze whether the variation p.Cys1281Tyr causes significant intracellular retention and further confirm that the cysteines and their integration into intrachain disulfide bonds plays a key role in the intracellular trafficking of the TG. Variants that introduce a premature stop by nonsense variants (stop-gained), shift the open reading frame (frameshift), or alter the two essential splice site nucleotides immediately to the left and right of each exon, that is, variants that can unequivocally impair protein, they were considered pathogenic. On the contrary, the deleterious effect caused by missense variants, as the p.Cys1281Tyr, needs greater precision. Previously, in our laboratory identify the patient II-4 of the C family as a compound heterozygous for p.Cys1281Tyr (originally published as p.Cys1262Tyr, not including the 19 amino acid signal peptide) and p.Tyr126* [Citterio et al., 2013] (Supplementary Figure 2). We also identify the variant p.Cys1281Tyr associated with previously identified c.5686+1G>T (Skipping of exon 30) variant [Pardo et al, 2008, 2009; Targovnik et al., 1995, 2001], in two siblings with CH (data not shown).

Human *TG* gene is a single copy gene of 268 Kb long that maps on chromosome 8 (8:132,866,958–133, 134,903; GRCh38 assembly) divided into 48 exons, encoding 8455 nt long mRNA sequence (of which 8304 nt correspond to coding sequences, NCBI: NM_003235.5.) [Citterio et al., 2021; Mendive et al., 2001; van de Graaf et al., 2001]. The monomeric human TG preprotein has a leader peptide of 19-amino acids followed by 2749-amino-acid polypeptide (NCBI: NP_003226.4 and Uniprot: P01266) [Holzer et al., 2016; Malthiéry & Lissitzky, 1987; van de Graaf et al., 2001]. The classical model of the primary structure of the human TG is organized into four regions (I, II, III and IV) [Holzer et al., 2016]. Region I comprises 10 of the 11 TG type 1 repeats, two modules called linker (residues 359 to 604) and hinge (residues 1211 to 1455) plus an N-terminal T_4 forming site. Region II contains 3 TG type 2 repeats and the 11th TG type 1 repeat, whereas region III contains all five TG type 3 repeats (Figure 4A). The fourth region is integrated of the cholinesterase-like (ChEL)

domain, between residues 2211 to 2735 of TG [Park et al., 2004; Swillens, 1986] and a C-terminal T_3 forming site (Figure 4A). Recently, Coscia et al. [2020] and Adaixo et al. [2022] reported the three-dimensional structure of human TG using a composite cryo-electron microscopy (cryo-EM) density map, at an overall resolution of 3.5 Å and 3.2 Å, respectively. Coscia et al. [2020] described the structure of deglycosylated TG from thyroid glands of patients with goitre as well as non-deglycosylated TG recombinant expressed in HEK293T cells and the atomic model of dimeric TG covers 93% of sequences. On the other hand, Adaixo et al. [2022] described the structure of the native, non-deglycosylated TG from healthy human thyroid glands and the atomic model of the dimeric TG covers about 90% of the molecule. Meanwhile, Kim et al. [2021] and Marechal et al. [2022] reported the structure of native bovine TG at an overall resolution of 2.6 Å and 3.3 Å, respectively. These models provide high resolution information on the structural organization of domains, hormonogenic, proteolysis and glycosylation sites, and formed disulfide bridges. Interestingly, Coscia et al [2020] proposes a new model of the primary structure of the human TG organized in five regions: N-Terminal Domain (NTD, positions 31–620), Core (positions 621–1210), Flap (positions 1211–1438), Arm (positions 1439–2186) and C-Terminal Domain (CTD, positions 2187–2768). CTD region contains the ChEL (dimer) domain type, positions 2187–2728) (Figure 4B). The complex architecture of the TG revolves around the central ChEL (dimer). However, the models proposed by Adaixo et al. [2022] and Kim et al. [2021] follow the general guidelines of the classical model for the TG monomer. The p.Cys1281Tyr variant is located in the hinge module of the region I in the classical model (Figure 4A), that corresponds to the M domain of the Flap region in the new model (Figure 4B).

We demonstrated that the p.Cys1281Tyr variant, causes intracellular retention in HEK293T cells. Previously, the multiple alignment analysis of the TG (*Homo Sapiens*, *Bos Taurus and Rattus Norvegicus*) sequences shows that the wild-type cysteine^{1281} is strictly conserved in the TG of three analyzed species, suggesting an important role of this amino acid for the structure of the TG and/or its correct functionality [Citterio et al., 2013]. On the other hand, the present study showed that 18 of

the 21 predictors were informative of a deleterious effect of the p.Cys1281Tyr variant and that 3D modeling showed that the presence of the tyrosine^{1281} clearly affects the structure of the TG. Consequently, in addition to not being identified in the gnomAD data set (v2.1.1 and v3.1.2) and the clear analysis of the C family pedigree (Supplementary Figure 2), we can affirm that the p.Cys1281Tyr in the TG is a variant pathogenic that is retained intracellularly and in association with the p.Tyr126* variant, located on the opposite allele, causing the thyroid disease in the patient II-4 of the C family [Citterio et al., 2013] (Supplementary Figure 2).

Wild-type cysteine^{1281} forming a disulfide bond with cysteine^{1249} (Supplementary Table 1). The cysteine^{1249} in human TG corresponds to cysteine^{1250} in rat [UniProt P06882; Citterio et al, 2020] TG. Each TG monomer contains 123 cysteine residues, one of which is located in the signal peptide (cysteine^{15}) (Supplementary Table 1) [Holzer et al., 2016; Malthiéry & Lissitzky, 1987; van de Graaf et al., 2001]. TG is folded through the putative formation of 61 intrachain disulfide bonds (Supplementary Table 1). TG type 1, TG type 2 and TG type 3 domains are cysteine-rich repeat modules that are covalently bound by intrachain disulfide bridges, whereas the ChEL domain contains six cysteine residues. These bridges are involved in the conformation of the tertiary structure of TG, adding structural stability, solubility and rigidity to the protein [Adaixo et al., 2022]. Loss of cysteine residues removes disulfide bonds, disrupting the normal TG architecture, causing a defect in protein secretion, and possibly preventing the interaction of hormonogenic donor and acceptor sites [Citterio et al., 2021]. We previously studied the possible pathogenicity of sixty-seven wild-type cysteine residues found in the gnomAD v2.1.1 data set and in patients with thyroid disease, using four “in silico” algorithms (PROVEAN, SIFT, Polyphen-2 (Hum Var) and Panther-PSEP) (Supplementary Table 1) [Pio et al., 2021]. The predictors exhibited putative pathogenicity in all the variants analyzed, classifying them in likely pathogenic group, with the exception of a single variant, p.Cys15Ser, located in the signal peptide, classifying it as likely benign (Supplementary Table 1). A detailed study of two unrelated CH patients and two siblings with adenomatous goiter and serum TG levels above

the normal range, revealed replacement of two conserved cysteines by arginine (p.Cys1264Arg, originally published as p.Cys1263Arg) and serine (p.Cys1996Ser, originally published as p.Cys1995Ser), respectively. Hishinuma et al. [2005] showed that goiter resulting from p.Cys1264Arg variant is associated with papillary thyroid cancer. Yoon et al. [2020] described anaplastic thyroid cancer in a 46-year-old woman with TDH associated with the p.Cys1264Arg variant of the TG. The p.Cys1264Arg variant is also localized in the hinge module/flap region, presumably disrupting a local disulfide bond with cysteine[1245]. Recently, Wright et al. [2021] have employed a multiplexed quantitative **A**ffinity **P**urification–**M**ass **S**pectrometry (AP-MS) platform coupled to **T**andem-**M**ass-**T**ag (TMT) labeling to define the TG proteostasis network interactome. They confirmed that p.Cys1264Arg is retained intracellularly while exhibiting increased interactions with chaperoning and oxidative protein folding pathway components.

On the other hand, Zhang et al. [2021] demonstrated that thyroxine production in TG defects by deleterious variants is synthesized from mutant TG retained intracellularly and released into the follicle lumen by dead thyrocytes. The released mutant TGs are cannibalized by the thyroid hormone biosynthetic machinery of surrounding living thyrocytes, which are responsible for iodinating them and synthesizing thyroxine. Consequently, we can hypothesize that in patients with biallelic missense cysteine's TG variants or associated with another pathogenic variant on the opposite allele, this cannibalization mechanism would partially compensate for the lack of TG secretion at the apical membrane/colloid interface.

In conclusion, we confirm that the p.Cys1281Tyr variant has a high degree of pathogenicity, causing intracellular retention in HEK293T cells. This study also constitutes a contribution to confirm the pathogenicity of TG missense variants through an algorithm that integrates the analysis of the familial genotype, the population frequency in the gnomAD database, the multiple alignment analysis, the use of a large group of predictors, 3D modeling of the mutant and its transient expression.

Figure legends

Figure 1. 3D structure of the p.Cys1281Tyr human thyroglobulin variant. A) The wild-type human thyroglobulin monomer is shown with its four regions according to the classical model, regions I, II, III and IV. Region I in yellow color, region II in violet color, region III in cyan blue color and region IV (corresponding to ChEL domain) in red color; delimited box shows the hinge domain, (also part of the region I) marked in blue color for its better visualization; black arrows show the cysteine^{1281}. B) The black arrow shows the mutated tyrosine^{1281} and open arrows shows the arginine^{1264}.The figure was rearranged to better visualize of amino acid variant.

Figure 2. 3D structure of the p.Cys1282Tyr bovine thyroglobulin variant (p.Cys1281Tyr in humans). Bovine thyroglobulin was used due to its better resolution at the observed structural area. A) The black arrow shows the wild-type bovine thyroglobulin cysteine^{1282} (cysteine^{1281} in humans), making disulfide bond with wild-type bovine thyroglobulin cysteine^{1250} (cysteine^{1249} in humans) (open arrow). The blue dotted lines we can visualize the nine hydrogen bonds in this delimited area. B) The black arrow shows the mutated bovine thyroglobulin tyrosine^{1282} (tyrosine^{1281} in humans), followed by the disruptions of the disulfide bound between the wild-type bovine thyroglobulin cysteine^{1282} and cysteine^{1250} (cysteine^{1281} and cysteine^{1249}in humans) (open arrow). C) The pink dotted lines indicate the occurrence of clashes in the presence of the mutated bovine thyroglobulin tyrosine^{1281}(tyrosine^{1281} in humans) (black arrow). The hydrogen bonds, 5, 6, 7, 8 and 9 in close association with the clashes could probably lost during the reaccommodation process.

Figure 3. Defective secretion of the prTG[p.Cys1281Tyr] clone. A) A schematic representation of the previously published cloning of the full-length rat thyroglobulin cDNA [Citterio et al., 2020] and the generation of the prTG[p.Cys1281Tyr] mutated clone by site-directed mutagenesis is shown. The sequence of the forward (3842G>AF) and reverse (3842G>AR) mutagenesis oligoprimers are shown.

B) HEK293T cells were transiently transfected with the prTG[p.Cys1281Tyr] and prTGwt plasmids and the cell lysates (C) and the culture supernatants (M) were analyzed by 7%-SDS-PAGE followed by immunoblotting with antibodies against thyroglobulin as indicated in Materials and Methods. Lane HEK293T is negative control. MK, Page Ruller Prestained Protein Ladder (10-180 KDa) (Thermo Fisher Scientific, Waltham, MA).

Figure 4. Structural organization of wild-type thyroglobulin protein and mutant p.Cys1281Tyr. A) Classical model of the primary structure of thyroglobulin of the putative p.Cys1281Tyr mutant. The schematic representation adapted from Holzer et al. [2016]; Malthiéry & Lissitzky [1987]; Mercken et al. [1985]; Molina et al. [1996], Parma et al. [1987]; van de Graaf et al. [2001] and Swillens et al. [1986]. Thyroglobulin signal peptide (SP), TG type 1, TG type 2, and TG type 3 repeating units, linker and hinge modules, spacers 1, 2 and 3, and acetylcholinesterase (ChEL) homology domain are drawn to scale and represented by boxes. The thyroglobulin monomer is organized into four framework regions (I, II, III, and IV). Shown are the sites of formation of N-terminal T_4 (coupling of a DIT^{149} donor with the DIT^{24} acceptor) and C-terminal T_3 (coupling of an MIT^{2766} on the antepenultimate residue of a TG monomer with the antepenultimate DIT^{2766} on the opposite monomer). B) New model of primary structure of thyroglobulin of the putative p.Cys1281Tyr mutant. Schematic representation adapted from Coscia et al. [2020]. Thyroglobulin signal peptide (SP) and TG type 1 (domains A, B, C, D, F, G, H, J, K, L and P), TG type 3 (domains Q, R, S, T and U), TG type 1-like (dimer, I domain), Ig-like (M and N domains), TNF/EGF/laminin-like fold (O domain), and acetylcholinesterase homology (ChEL (dimer, V domain), are drawn to scale and represented by boxes. The thyroglobulin monomer is organized into five structural regions: N-Terminal Domain (NTD), Core, Flap, Arm, and C-Terminal Domain (CTD). Amino acids are numbered, including the 19 amino acids of the signal peptide, following NCBI numbering: NP_003226.4 and UniProt P01266.

Acknowledgements

M.G.P. and M.F.M. are research fellows of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET). H.M.T., K.G.S. and C.M.R. are established investigators of the CONICET.

Statement of Ethics

The studies involving human participants were reviewed and approved by the Ethical Committee of the Faculty of Pharmacy and Biochemistry of the University of Buenos Aires (CEICFFyB, No. 1094). Written informed consent was obtained from the parents of the children involved in this study.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

This study was funded by grants from the Fondo para la Investigación Científica y Tecnológica (FONCyT-ANPCyT-MINCyT, PICT-2018-02146 to H.M.T.), CONICET (PIP 2021-11220200102976CO to C.M.R.) and Universidad de Buenos Aires (UBACyT 2020-20020190100050BA to C.M.R.).

Credit Author Statement

M.G.P. contributed to Sanger sequencing, performing structural modeling analysis, site-directed mutagenesis, transfection, and TG protein secretion analysis. E.A. contributed to TG protein secretion analysis. M.B.M. and G.S. were involved in the recruitment of patients and acquisition of clinical data and blood samples. M.F.M. and K.G.S. contributed to bioinformatic analysis. C.M.R. contributed to the acquisition of funds and the design of the study. HMT contributed to bioinformatics predictive

analysis, funding acquisition, study conception and design, and writing of the article. All authors critically reviewed and participated in manuscript revision and approved the final draft.

Data Availability Statement

Data and material are available from the authors upon request.

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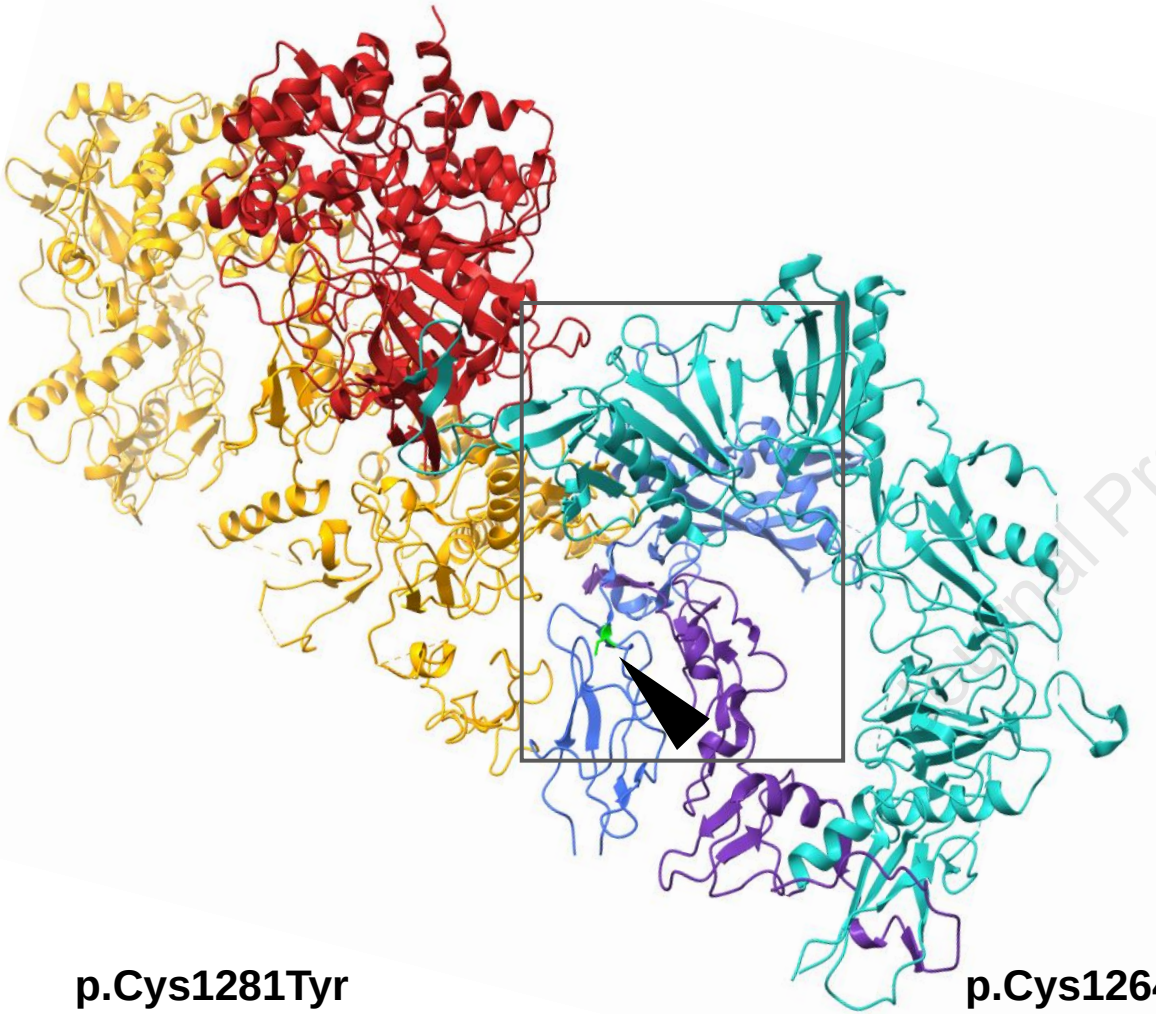

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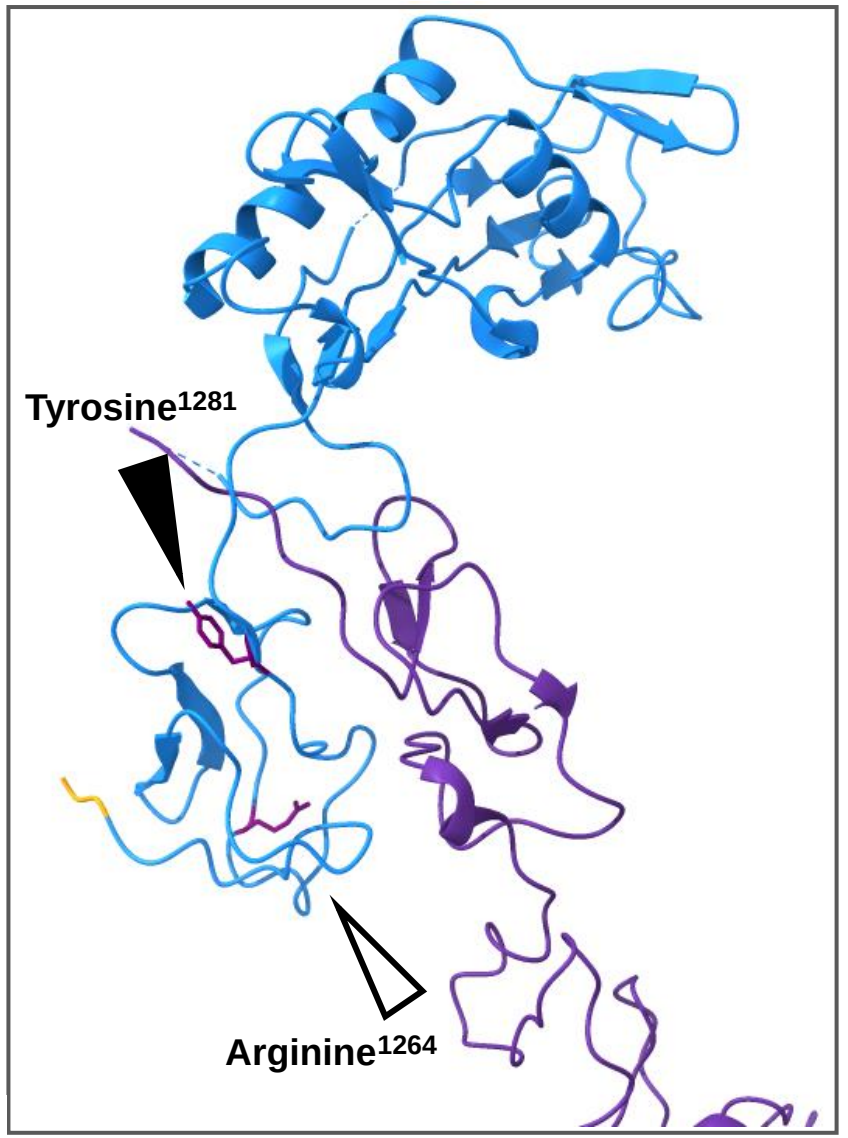
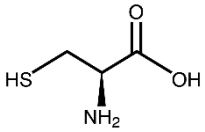


p.Cys1281Tyr

Cysteine



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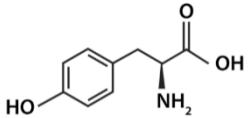

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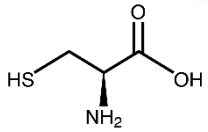

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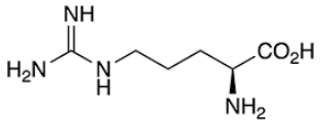

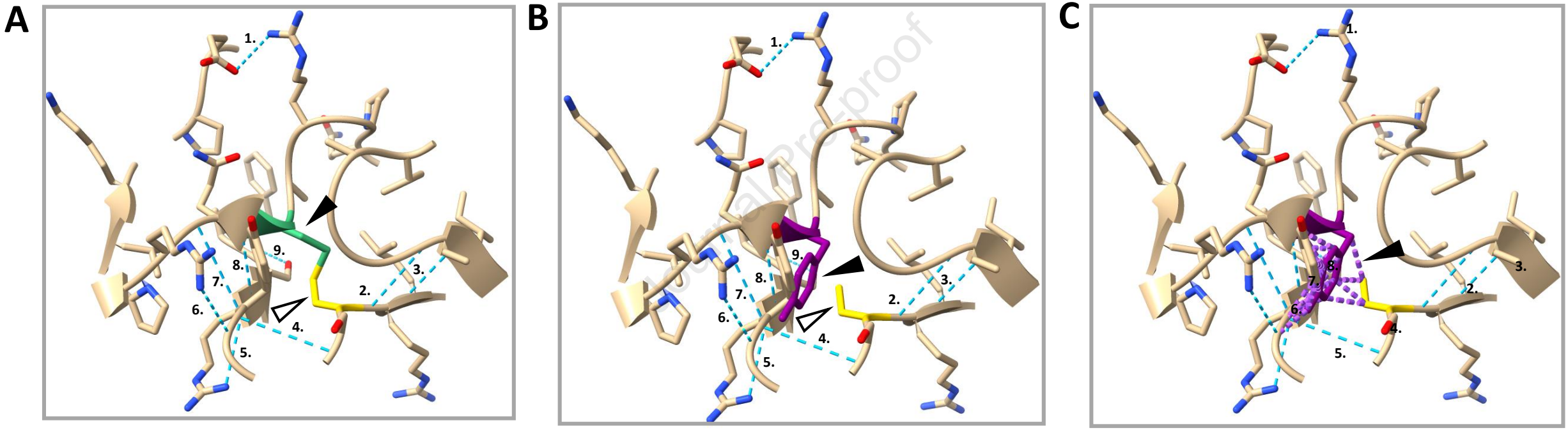
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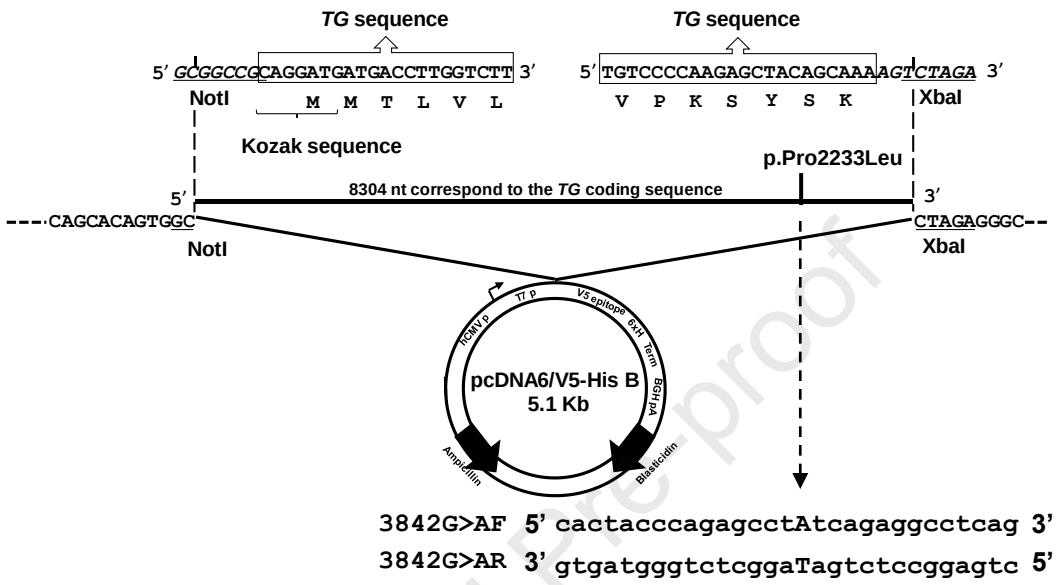

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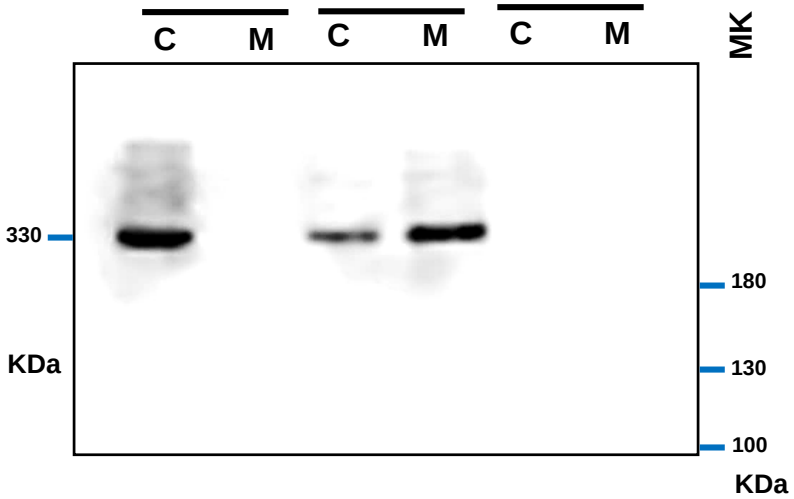

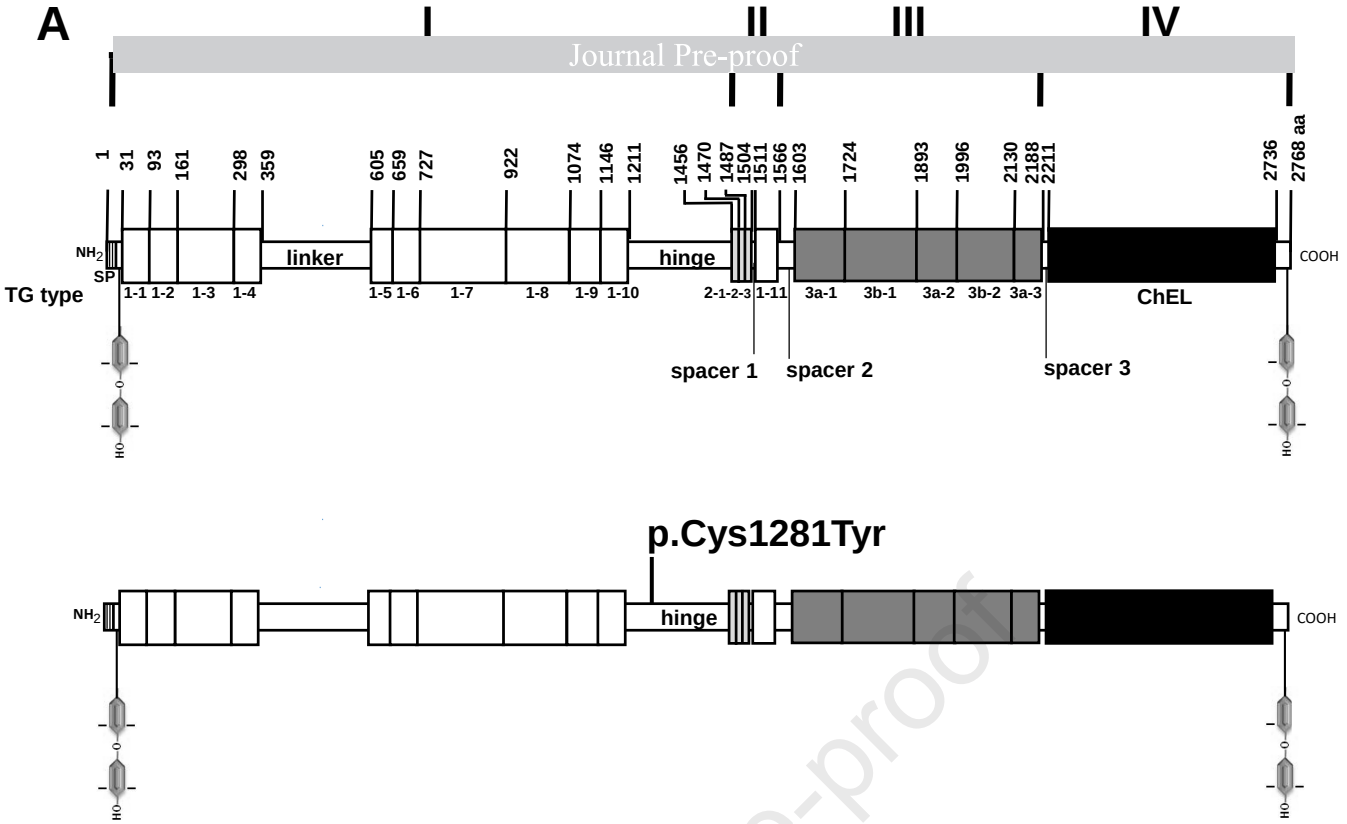

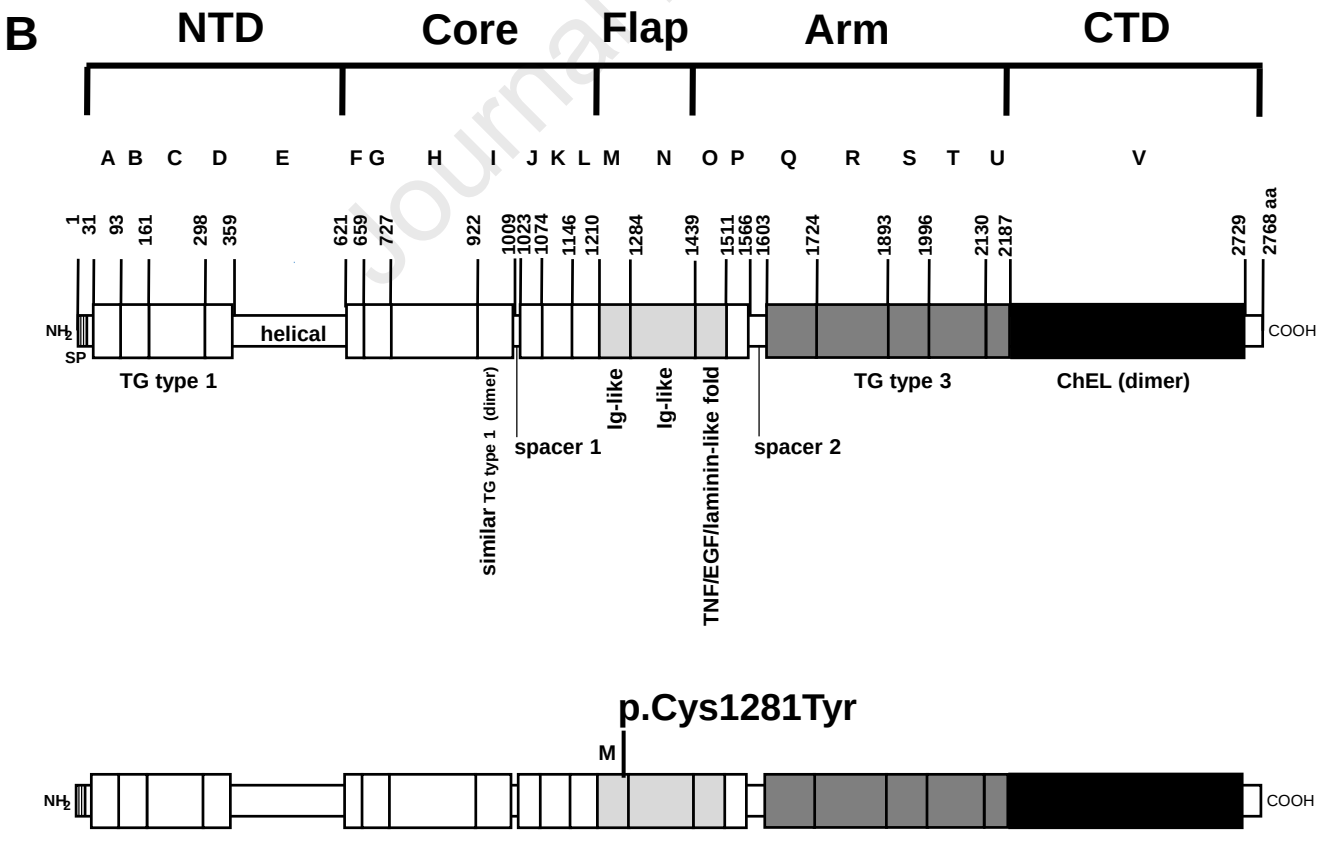

Highlights

- We report bioinformatic and expression studies of the p.Cys1281Tyr thyroglobulin (TG) variant.
- *"In silico"* algorithms predict a deleterious effect of this TG variant.
- The full-length 3D model showed rearrangement of the TG structure.
- Transient expression analysis indicated that p.p.Cys1281Tyr causes intracellular retention.
- Our results confirm that cysteines plays a key role in the intracellular trafficking.

Declarations of interest: none