Original Article

Novel Mutation (T273R) in Thyroid Hormone Receptor β Gene Provides Further Insight into Cryptic Negative Regulation by Thyroid Hormone

(thyroid hormone receptor β / negative regulation / negative feedback regulation / syndrome of resistance to thyroid hormone / RTH / T273R)

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Abstract. Production of thyroid hormone is precisely regulated in a negative feed-back mechanism that depends critically on thyroid hormone receptor β (TRβ). This mechanism decreases production of thyrotropin-releasing hormone (TRH) and thyrotropin (TSH) in the hypothalamus and pituitary gland in response to high levels of circulating thyroid hormones (TH). Despite the wealth of accumulated knowledge, it is still not clear how exactly this negative regulation is executed. The syndrome of resistance to thyroid hormone (RTH), in which the levels of TH are not properly sensed, represents naturally occurring situations in which molecular components of this regulation are displayed and may be uncovered. TRβ, which is central to this regulation, is in the majority of RTH cases mutated in a way that preserves some functions of the receptor. Approximately 150 different mutations in TRβ have been identified to date. Here, we hypothesized that additional pathogenic mutations in TRβ are likely to exist in human population and analysed clinical cases with suspected RTH. In keeping with our prediction, analysis of 17 patients from nine families led to identification of four presumed pathogenic mutations of TRβ, including a previously unknown mutation, T273R. This suggests that threonine 273 is likely to be critical for the normal function of TRβ, possibly due to its role in helix 12 mobility and interaction with coactivators, and thus supports the concept that TRβ-dependent trans-activating function is necessary for the inhibition of TRH and TSH expression in response to elevated levels of TH.

Received November 9, 2016. Accepted February 13, 2017.

This work was supported by the European Regional Development Fund "BIOCEV – Biotechnology and Biomedicine Centre of the Academy of Sciences and Charles University in Vestec" (CZ.1.05/1.1.00/02.0109) (Start-Up Grant to the group Structure and Function of Cells in Their Normal State and in Pathology – Integrative Biology and Pathology (5.1.10)) and the LQ1604 National Sustainability Programme II (Project BIOCEV-FAR) from the Ministry of Education, Youth and Sports of Czech Republic; grant PRVOUK-P27/LF1/1 from Charles University, grants SVV 260023/2014, SVV260149/2015, SVV260257/2016 and SVV 260377/2017 from Charles University.

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Abbreviations: ADHD – attention deficit/hyperactivity disorder, CoA – coactivators, CoR – corepressors, DBD – DNA-binding domain, EGF – epidermal growth factor, LBD – ligand-binding domain, nTre – negative TRE, PCR – polymerase chain reaction, RTH – resistance to thyroid hormone, T_3 – triiodothyronine, T_4 – thyroxine, TH – thyroid hormones, TRβ – thyroid hormone receptor β, TRE – thyroid hormone response element, TRH – thyrotropin-releasing hormone, TSH – thyrotropin.

Introduction

The molecular actions of thyroid hormones (TH) are mostly mediated via thyroid hormone receptors (TRs), expressed in all vertebrates as at least three unique isoforms originating in two genes (Sap et al., 1986; Weinberger et al., 1986). The *THRB* gene locus on the 3rd chromosome produces, via differential transcription start, two isoforms: TRβ1 and TRβ2 (Williams, 2000). While TRβ1 is widely expressed, TRβ2 is tissue-specific and is present predominantly in the hypothalamus and anterior pituitary (Hodin et al., 1990). All TRs share high structural homology with an optional N-terminal A/B domain, a central DNA-binding domain (DBD), a hinge region and a C-terminal ligand-binding domain (LBD) (Lazar, 1993). Apart from binding of T_3, LBD is also responsible, in cooperation with the A/B domain

(Langlois et al., 1997), for the recruitment of coactivators (CoA) and corepressors (CoR) (Cheng et al., 2010).

The majority of human genes are regulated positively by TRs. These genes are silenced in the absence of triiodothyronine (T_3) and over-activated in its presence. Apart from these, there are also genes regulated by T_3 in a negative manner, among others thyrotropin-releasing hormone (TRH) (Hollenberg et al., 1995), thyrotropin (TSH) α and β subunits (Burnside et al., 1989, Wondisford et al., 1989), epidermal growth factor (EGF) (Thompson et al., 1992) and keratin genes (Tomić-Canić et al., 1996). In some of these genes, distinct negative thyroid hormone response element (nTRE) sites have been identified (Hollenberg et al., 1995). The mechanism of this reverse regulation remains somewhat enigmatic. One hypothesis describes this effect being mediated by inactivation of corepressor (Yang et al., 1999) and activation of coactivator (Yang and Privalsky, 2001) binding, followed by reversed action of TR-interacting corepressors and coactivators (Tagami et al., 1997, 1999). The exact role of TRβ2 isoform in this negative regulation is not clear; however, in certain cases it seems to be a crucial mediator of the trans-regulating effect (Langlois et al., 1997; Yang and Privalsky, 2001). Reversed regulation by T_3 is specifically important in the negative feedback regulation in the TRH-TSH-T_3 axis.

Various mouse models have been produced to determine which TRβ domains and specific amino acid residues play the dominant role in mediating the target gene expression inhibition, largely by observing its most apparent phenotypical consequence, the TRH-TSH-T_3 axis deregulation (Dumitrescu and Refetoff, 2013). While these animal models provided much valued information, another approach to establish the structural basis for negative TRβ regulation can be exploited by studying the consequences of *THRB* gene disturbances in an organism observable in a different, arguably more detailed way, the human. A specific nosological unit of the syndrome of resistance to thyroid hormone β (RTH β) offers an opportunity to observe phenotypical changes originating from individual mutations in the *THRB* gene.

Due to the variegated normal expression of the distinct TR isoforms in individual tissues, the phenotype of RTH β has the clinical hallmarks of both insufficient and excessive thyroid hormone action. Among the most prevalent are goitre formation, behavioural and cognitive disturbances and sinus tachycardia (Dumitrescu and Refetoff, 2013).

In most of RTH β patients, a heterozygous single-nucleotide substitution was found, resulting in miss-sense translation of a single incorrect amino acid. All of the mutations leading to RTH β were localized in the last four exons of the *THRB* gene, concentrated in mutational hot-spots separated by "cold regions", often in CpG dinucleotides. These exons translate into the aforementioned hinge region and LBD, with the mentioned "cold regions" projecting into regions of the molecule important for dimerization, DNA binding and interaction with CoR (Dumitrescu and Refetoff, 2013).

It has been shown that a complete single allele deletion of *THRB* gene has no phenotype, which is not caused by overexpression of the remaining allele (Hayashi et al., 1993). This indicates that the clinical features of RTH β are the result of a dominant negative effect of the mutated isoform (Chatterjee et al., 1991). The mutated TRβ has preserved DNA binding and thus occupies TREs; however, it is not capable of T_3 binding-induced transregulation either due to its inability to bind T_3 (Refetoff, 2008), its decreased association with CoA (Collingwood et al., 1998), or its increased affinity to CoR (Yoh et al., 1997). It results in formation of non-functional homo- and heterodimers refractory to T_3, competitively hindering both positive and negative regulation of expression of the respective TH-regulated genes. The impaired negative regulatory function, which is in many cases affected predominantly, is responsible for the pathognomic trait of resistance to thyroid hormone (RTH), the elevated free serum T_3 and thyroxine (T_4) levels with non-suppressed TSH levels (Clifton-Bligh et al., 1998). Patients harbouring mutations exhibiting such phenotype are, in fact, well describable subjects, which upon identification of the causal mutation documents the structural relevance of individual amino acid residues. These residues can be moreover related to molecular functions of the individual structural units of TRβ, conversely identifying the molecular mechanisms involved in the disrupted negative regulation by TRs.

In this study we attempted to screen suspected cases of RTH based on the presence of the described biochemical traits of the disturbed negative feedback mechanism of thyroid function. This could grant further insight into the functional relevance of individual structural elements (amino acids, helices, domains) in the cryptic negative regulation by TRβ.

Material and Methods

All patients with suspected RTH were clinically examined by cooperating specialized endocrinologists. The biochemical assays for TSH, T_3 and T_4 serum levels were performed by various licensed clinical laboratories. Written informed consent for a genetic study was signed by all patients or their legal guardians, respectively. The Ethics Committee of the First Faculty of Medicine, Charles University and the General University Hospital in Prague (Approval of June 21, 2012, project title: "Genetic and Molecular Characterization of the Regulation by Thyroid Hormone", Chairman of the Committee Josef Šedivý, MD, PhD) approved the project execution.

Peripheral venous blood samples were obtained from the patients and transported to the clinical on-site laboratory. Genomic DNA was isolated from the blood samples using an automated magnetic bead technique. Four terminal exons of the *THRB* gene were amplified by polymerase chain reaction (PCR) using Q5® High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA) and the primer oligonucleotides were designed in

Table 1. Sequences of used primers

Primer sequences	Forward	Reverse
Exon 7	5‘-gcatctgtgtgccttgtctc-3‘	5‘-tgaggtagaaaacactggcata-3‘
Exon 8	5‘-caacttcttcatttaaatctttctttt-3‘	5‘-attcctggaaactgatgaaactat-3‘
Exon 9	5‘-tgttgttcctgactggcatt-3‘	5‘-agcgctagacaagcaaaagc-3‘
Exon 10	5‘-taaaggcctggaattggaca-3‘	5‘-ggcaatggaatgaaatgaca-3‘

concordance with the sequences described previously (Rocha et al., 2007, Table 1). To prevent an amplification-induced sequencing error, two sets of reactions were prepared for each exon amplification of each patient. PCR reaction conditions were set according to the user manual, with 30 amplification cycles, annealing temperatures determined for each primer pair via the manufacturer’s online calculation tool (NEB Tm calculator, http://tmcalculator.neb.com) and a universal extension time of 30 s. PCR reaction mixes were purified using the column membrane purification method Macherey-Nagel NucleoSpin® gel and PCR clean-up (Macherey-Nagel GmbH & Co. KG, Düren, Nordrhein-Westfallen, Germany).

Samples were sequenced by the commercial sequencing company GATC Biotech AG (Cologne, Germany). Each exon was sequenced from two distinct PCR reaction mixes with each of the primers used to amplify exon regions.

TRβ orthologues from OMA database (http://omabrowser.org) were aligned with MUSCLE (Edgar, 2004). Additional sequences were obtained by BLAST searches with this alignment (NCBI, Bethesda, MD) and included. Representative examples were selected from about 100 diverse sequences. The structures were displayed and analysed with BrowserPro (MolSoft L.L.C., San Diego, CA) (http://www.molsoft.com).

Results

In total, 17 patients within the period of three years had been referred to us for genetic analysis due to clinical and biochemical signs indicative of RTH as a part of a pilot study for establishing an RTH diagnostic protocol. Out of these, four families (of 4, 4, 2 and 2 members respectively) constituted 12 patients with the remaining five being sole probands. All the patients were referred from a wide area in the vicinity of Prague, Czech Republic by their respective endocrinologists.

Out of these, the investigation of mutational hot-spots of the *THRB* gene of one family of four and four individually affected probands observed no mutation and a biochemical diagnosis attempt was recommended as described in Dumitrescu and Refetoff (2013). In the mentioned loci, in one family of four, a previously described mutation, K443E (A/G mutation in nucleotide 1327 of TRβ1 CDS) (Sasaki et al., 1992), was observed and in one family of two, a different mutation, F459L (T/C in 1375) (Mitchell et al., 2010), was found. One investigated patient had previously observed mutation Y321C (A/G in 962) (Adams et al., 1994). Sequencing of the TRβ loci of the remaining family of a mother and a daughter yielded a novel mutation, T273R (C/G in 818), to our best knowledge not yet described. All of these mutations were observed in a heterozygous conformation.

The proband mother is a 37-year old woman dominantly affected by attention deficit/hyperactivity disorder (ADHD). The patient has suffered dominantly by feeling of distractibility, lack of concentration and increased tiredness resulting in educational and professional challenge and secondary psychological disorders, namely clinical depression. Therapy with methylphenidate and atomoxetine resulted in improvement in school results and subjective psychological state. Associated clinical depression has been managed with continuous escitalopram administration. Increased sweating, as well as a subjectively decreased tolerance of cold, were mentioned by the patient. Dysregulation of peripheral circulation was described, with vasodilatation and stasis in hand blood vessels in the cold. Furthermore, significant non-infectious spontaneous vaginal discharge has been consistently present since puberty.

During a routine endocrinological investigation, disturbance in the thyroid hormone regulatory axis was discovered and she was referred to a specialized endocrinologist, where detailed biochemical investigation of the thyroid function was performed. It revealed mildly elevated serum free T_4 levels (29.38 pM) with non-suppressed TSH levels (2.210 mIU/l). Concurrently, elevated titres of anti-thyreoperoxidase (anti-TPO, > 1300 kIU/l) and anti-thyroglobulin (anti-hTGc, 204 kIU/l) antibodies were observed. Thereafter, her thyroid gland was ultrasonographically examined, with finding a sole isoechogenic cavitated node of 1.4 ml and benign cytological characteristics. Goitre was not present and the patient did not exhibit any further signs of thyroid disease associated with RTH such as sinus tachycardia, growth and developmental delay, weight changes and hearing impairment. In the last two years, the patient has been administered levothyroxine (50 μg, 5 times per week) with positive effect on exhaustion, apathy and concentration disturbance.

Genetic investigation revealed a family history of thyroid dysfunction-associated symptoms in the father of the patient. Despite the paucity of preserved clinical evidence, the proband patient ascertains the relatedness of the father’s symptoms to her own. He also exhibited the pathognomic biochemical traits of RTH. He deceased aged 56 due to a cardiac tamponade of undis-

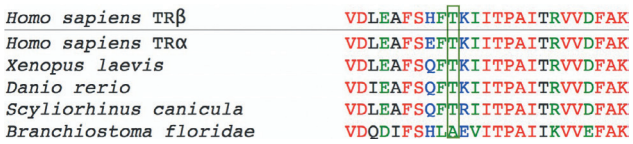

Fig. 1. Evolutionary conservation of the affected Thr273
Amino acid sequence alignment of TRβ proteins from various chordate orthologues and human paralogue TRα. The concerned amino acid residues corresponding to position 273 of human TRβ is highlighted. The threonine residue is conserved absolutely among higher chordates (Crania) as exemplified on mammals, amphibians (*X. laevis*), bony fish (*D. rerio*) and shark (*S. canicula*); only lower chordates such as Cephalochordates lancelets (*B. floridae*) are divergent. UniProt/NCBI identifiers from top to bottom: P10828.2, P10827.1, NP_001081250.1, NP_571415.1, ABS11251.1 and ABS11249.1.

closed origin. The patient's mother is living without any signs of thyroid dysfunction. The proband patient has one four-year-old daughter in independent observation by a paediatric endocrinologist.

The initial suspicion for the diagnosis of RTH in the daughter of the proband was based on a biochemical investigation performed during a hospitalization due to a solitary occurrence of febrile seizures at the age of 1. Consistently with the mother's biochemical features, the daughter's serum free T_4 levels were highly elevated (41.97 pM) with non-suppressed TSH levels (4.97 mIU/l). Since then, the daughter has been under under observation for the suspected RTH. She has exhibited slightly impaired thriving and the mother described disturbances in the sleep habits and irregular loose stools. Ultrasonography of the thyroid gland at 33 months of age revealed a diffuse goitre (6.5 ml of combined volume). Clinical investigation revealed no other signs of thyroid dysregulation, she began to progressively catch up in growth (up to 50th percentile) with BMI remaining low (20th percentile). Clinical hearing examination revealed no pathology.

Inspection of the available TRβ orthologues and paralogues in the UniProt and NCBI databases revealed very strong conservation of the position corresponding to T273 in evolution. Figure 1 illustrates the conservation on selected representative examples. Figure 2 shows the position of T273 in the crystal structure (Kojetin et al., 2015) of TRβ LBD with a bound ligand and a fragment of CoA. T273 is located in helix 2 tightly packing against the loop between helices 11 and 12. It does not interact with the ligand.

Discussion

Among the symptoms exhibited by both patients are thyroid irregularities, hyperactivity disorder and specific biochemical features of thyroid hormone insensitivity, which are all typical of patients suffering from the syndrome of RTH. Although a dichotomy into a pitu-

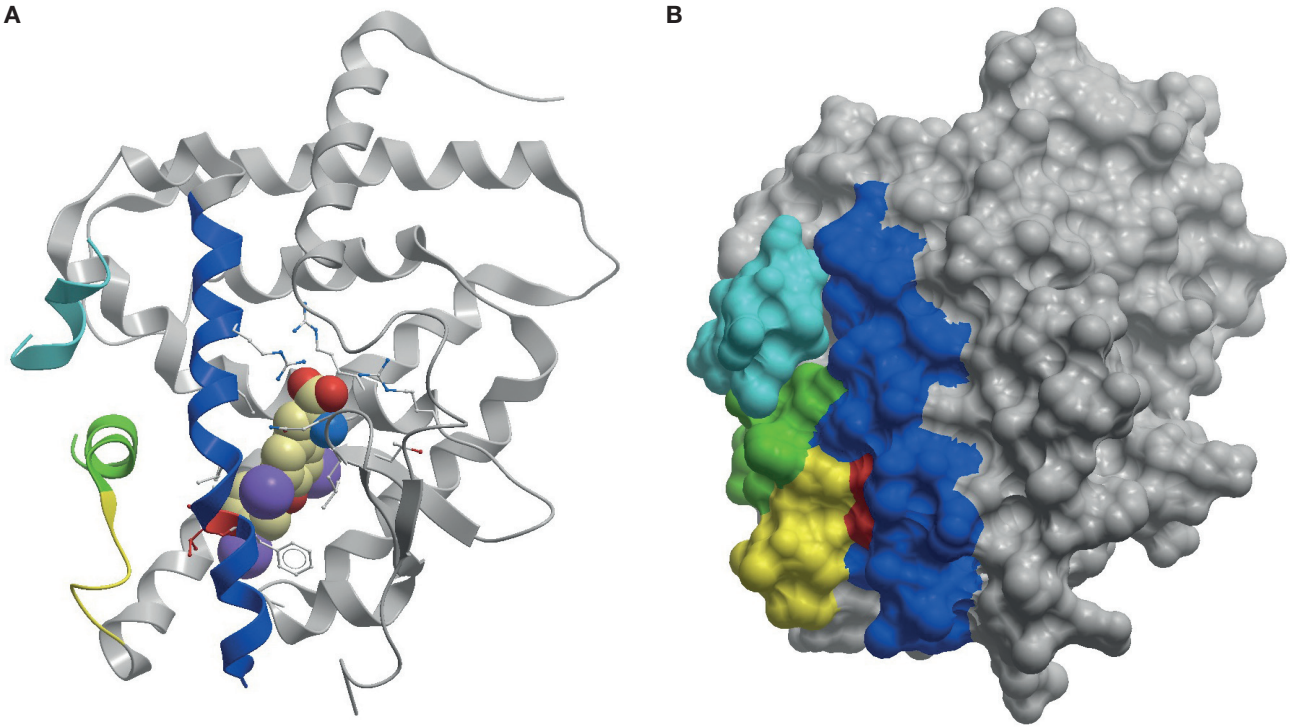

Fig. 2. Structure of TRβ with localized T273
Position of the mutated T273 (red) in the 3D structure (Protein Data Bank identifier 4zo1). Colours highlight the segments affected by the mutation: blue – helix 2, green – helix 12, yellow – loop between helices 11 and 12, cyan – fragment of nuclear receptor coactivator 2. **A.** Overall structure (ribbon) with the side chains (ball and stick) interacting with the ligand 3,5,3'-triiodothyronine (space filling). **B.** Surface presentations emphasizing the segment packing.

itary-specific and asymptomatic generalized form is shown to be pathophysiologically obsolete, it can be stated that in the case of our patients, significant, if moderate clinical presentation of both hypo- and hyperthyroid disorder is present (Beck-Peccoz and Chatterjee, 1994). The lack of another typical symptom of thyroid axis dysregulation, the sinus tachycardia, appears to be consistent in both patients; however, the enigmatic lethal cardiac tamponade in father of the proband could be postulated to be connected with affected resting heart rhythm. The daughter seems to be exhibiting slightly delayed growth, arguably also attributable to RTH. Furthermore, hypercholesterolaemia and cholelithiasis are shown to be more frequently prevalent in hypothyroidism (Laukkarinen et al., 2007), which might be hypothesized to be connected with the negative symptoms of the disease.

Due to the presented clinical and biochemical phenotype consistent with RTH β, its hereditary quality, discovered solitary heterozygous mutation T273R in the *THRB* gene and this novel mutation's characteristics, it can be presumed with high degree of confidence that it is the causal entity of the patients' syndrome.

The significance of T273 can be inferred from its evolutionary conservation (Fig. 1). Clear TR orthologues exist in all genomes of chordates except for tunicates. Two paralogues evolved in all known craniates and the threonine corresponding to the position 273 on human TRβ is strictly conserved in all of them. The divergence appears only in genomes of craniates with just one paralogue.

The newly discovered heterozygous cytosine to guanine mutation resides in the 8th exon of *THRB* gene in the locus 818. It results in a miss-sense translation of arginine in place of threonine (T273R in TRβ1) in the LBD of all TRβ isoforms. The mutated locus lies within the mutational cluster 3, however, in a sub-region, where a natural mutation was previously not described (codons 268-276) (Dumitrescu and Refetoff, 2013). The codon 273 of wild-type TRβ encodes the amino acid threonine, which is a part of helix 2 of the LBD. In the crystal structure, it is however not oriented in the inward direction to the T_3-binding pouch and threonine's polar hydroxyl-group is not interacting with T_3. It is in close proximity to the loop connecting helices 11 and 12, which is shown to be crucial for CoA recruitment (Barettino et al., 1994; Tone et al., 1994).

While most of the described mutations appear to impair T_3 binding (Adams et al., 1994; Hayashi et al., 1995; Collingwood et al., 1998), there is a subgroup of natural mutations, where the weakening or abolition of T_3 binding does not explain the severely impaired transactivating function of the TR and thus cannot correlate with clinical features of the syndrome (Hayashi et al., 1995; Collingwood et al., 1998). Our patient's T273R mutation appears to belong to this subgroup. T273 is located in helix 2 and does not interact with the ligand (Fig. 2A). It is almost completely buried and packs tightly with the loop between helices 11 and 12. The loop orients helix 12 for interaction with CoA (Fig. 2B, Kojetin et al., 2015). The mutation to arginine introduces a bulkier and positively charged side chain within these inner interfaces and CoA. A similar mutation described previously (T277A, Collingwood et al., 1998) has been shown to impair the T_3-dependent recruitment of CoA and its consequence predicted (without the knowledge of the 3D interaction with CoA) to be due to its intimate spatial relationship to helix 12. Although T273 is more distant from helix 12 than T277, its orientation is similar, and the known crystal structure confirms the close proximity to the loop and indirectly to helix 12 and CoA (Fig. 2).

Moreover, the mutation T273A has previously been identified in thyroid papillary carcinoma samples, among other mutations in *THRB*, but also, less frequently, in *THRA* genes. These mutations, including T273A, have been postulated to play an important role in the tumorigenesis of thyroid papillary carcinomas. The association of mutation T273A in TRβ with RTH is thus indicative of the importance of this residue for undisturbed function of TRβ (Puzianowska-Kuznicka et al., 2002).

Conclusions

We report novel mutation T273R in the *THRB* gene of patients exhibiting signs of the syndrome of resistance to thyroid hormone. We propose that the trans-activating function of TRβ is likely dominantly hindered in these patients. This suggests that TRβ-mediated activation is responsible for the negative feedback regulation of TH release. Identification of additional mutations in the *THRB* gene with a direct link to the general biological function of TRβ could, in addition to the clinical benefits of the diagnosis for the patient, broaden our understanding of the function of TRβ at the molecular level.

Acknowledgement

Authors thank Dr. Zdeněk Kostrouch for advice and Dr. T. Grimmichová, Dr. E. AlTaji, Dr. Z. Límanová, Dr. I. Plášilová and Dr. Z. Šumník for cooperation in case gathering and blood sample harvesting.

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