CHAPTER 2

Role of Leucine 341 in Thyroid Hormone Receptor Beta Revealed by a Novel Mutation Causing Thyroid Hormone Resistance

Karn Wejaphikul, Stefan Groeneweg, Prapai Dejkhamron, Kevalee Unachak, W. Edward Visser, V. Krishna Chatterjee, Theo J. Visser, Marcel E. Meima, Robin P. Peeters

*Extended version of **Thyroid.** 2018;28:1723-726.*

http://hdl.handle.net/1765/119488

2

Abstract

Background: Leucine 341 has been predicted from crystal structure as an important residue for thyroid hormone receptor β (TRβ) function, but this has never been confirmed in functional studies. Here, we verify the role of Leu341, driven by the identification of a novel L341V mutation in a 12-year-old girl with resistance to thyroid hormone β (RTHβ).

Methods: Genomic DNA was sequenced for mutations in the *THRB* gene. A novel L341V mutation as well as three artificial mutations (L341A, L341I, and L341F) were modeled in the wild-type (WT) T3-bound TRβ1 crystal structure. T3 binding affinity and transcriptional activity of the mutants were determined and compared with WT TRβ1.

Results: A heterozygous missense mutation in *THRB* (c.1021C>G; p.L341V) was found in a patient presented with diffuse goiter, tachycardia, and high serum FT4 and FT3 with non-suppressed TSH, indicative of RTHβ. Structural modeling of this mutation showed altered side-chain orientation and interactions of T3 with receptor. This was confirmed by *in vitro* studies demonstrating reduced affinity for T3 and impaired transcriptional activity of TRβ1-L341V. In addition, substitution of Leu341 by an alanine (A), isoleucine (I), or phenylalanine (F) reduced receptor function to various degrees, depending on their side-chain size and orientation and thus ability to maintain important structural interaction.

Conclusion: Leu341 has a critical role in T3 binding and hence TRβ function, and its mutation results in the clinical phenotype of RTHβ.

Introduction

Thyroid hormone (TH) is crucial for normal growth, development and metabolism. It is widely accepted that TH predominantly mediates its effects via transcriptional regulation of genes by binding of the active hormone, triiodothyronine (T3), to nuclear thyroid hormone receptors (TRs). Three functional TR isoforms, i.e., TRα1, TRβ1 and, TRβ2, are encoded by two different genes, *THRA* on chromosome 17 and *THRB* on chromosome 3 (1). The expression of TRs varies among tissues. TRα1 is mainly expressed in the brain, bone, heart, intestine, and skeletal muscle, whereas TRβ1 is principally expressed in the liver, kidney, and thyroid gland. TRβ2 is predominantly expressed in the retina, cochlea, as well as the hypothalamus and pituitary, where it plays a crucial role in a negative feedback control of the hypothalamic-pituitary-thyroid (HPT) axis.

Mutations in the *THRB* gene cause resistance to thyroid hormone β (RTHβ), which was first described in 1967 (2) and the first mutation was subsequently identified in 1989 (3). The estimated incidence is approximately 1:40,000 live births (4,5). The biochemical characteristics are elevated T4 and T3 with non-suppressed TSH concentrations because of impaired TRβ2 function in hypothalamus and pituitary, which consequently alters negative feedback control. The clinical phenotype is variable and may include goiter, tachycardia, and learning disability with or without hyperactive behavior.

RTHβ is usually inherited in an autosomal dominant fashion (6,7). Single nucleotide substitutions resulting in amino acid replacement are more common than frameshift or nonsense mutations that lead to premature protein truncations (8-10). These mutations mainly locate in 3 CpG transition and CG rich cluster regions (cluster 1; codon 426-461, cluster 2; 310-353, and cluster 3; codon 234-282) which encode the ligand binding domain (LBD) and the hinge region of TRβ protein (6). Therefore, mutations commonly impair affinity for T3 and consequently reduce transcriptional activity of the receptor.

The affinity for T3 of TRβ is determined by the interactions between the T3 molecule and the amino acid residues that form the ligand-binding cavity (11,12). For instance, crystallization of the TRβ protein and subsequent *in vitro* studies revealed that Arg282 and His435 play a crucial role in hormone binding (13-17). Another residue that was found to line the ligand binding pocket is Leu341 (18,19). A proline substitution at this position (L341P) has previously been described in RTHβ patients, supporting the importance of this residue (20,21). However, functional studies on the role of this Leu341 for the function of TRβ1 have not yet been established.

In this study, we describe a 12-year-old girl with RTHβ caused by a novel L341V mutation in TRβ. *In silico* studies suggest altered T3 binding of TRβ1-L341V which is confirmed by reduced affinity for T3 and impaired transcriptional activity in *in vitro* studies. In addition, substituting Leu341 with other non-polar amino acids also impairs receptor function to various degrees, depending on their side-chain size and orientation and thus ability to maintain

important structural interactions. These findings emphasize the functional importance of Leu341 for TRβ activity.

Materials and methods

Clinical and genetic assessment

The index patient was referred to our institute because of poorly controlled Graves' disease. Thyroid function tests were evaluated after one month of methimazole (MMI) withdrawal using an electrochemiluminescence immunoassay kit (Roche Diagnostic, Mannheim, Germany). Informed consent was obtained from the parents of the index patient. The study was approved by the Medical Ethics Committee of the Faculty of Medicine, Chiang Mai University, Thailand.

Genomic DNA was extracted from peripheral blood leukocytes by QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany). Exons 7-10 of the THRB gene [GeneBank: NM_000461.4], including exon-intron boundaries, were amplified (see Supplementary Table S1 for primers). Sequencing was performed as described previously (22). The exon carrying the mutation was re-amplified and sequenced to exclude a PCR error.

In silico prediction of mutant TRβ1 function

YASARA Structure Software (YASARA Bioscience GmbH, Vienna, Austria) (23) was used to model the TRβ1-L341V patient's mutation and three artificial mutants (L341A, L341I and L341F) into a T3-bound wild-type (WT) TRβ1 crystal structure (PDB-ID: 3GWS) (24) using the side-chain substitution tool. Side-chain orientations were optimized using SCWALL (Side-Chain conformations With ALL available methods) (25,26), after which the final models were minimized without further constraints. All images were created using YASARA Structure and Pov-Ray v3.6 software (www.povray.org).

DNA constructs and mutagenesis

The human TRβ1 cDNA was amplified and subcloned into the *EcoRI* and *XbaI* sites of the pcDNA3 expression vector fused at the 5'-end to the sequence encoding the FLAG-epitope tag and downstream of an optimized Kozak sequence (see Supplementary Table S1 for primers). The TRβ1-L341V patient's mutation (c.1021C>G) and three artificial mutants, including L341A, L341I and L341F, were introduced using the QuickChange II Mutagenesis kit (Agilent Technologies, Amstelveen, The Netherlands) according to manufacturers' protocol (see Supplementary Table S1 for primers). Sequences of mutant constructs were confirmed by Sanger sequencing.

[^{125}I]T3 competitive binding assay

Human FLAG-tagged TRβ1 WT and mutant (L341V, L341A, L341I and L341F) receptor proteins were synthesized in reticulocyte lysate using the TnT® T7 Quick Coupled Transcription/Translation System (Promega, Leiden, The Netherlands). The protein lysate was incubated with 0.02 nM of [^{125}I]T3 in 0.5 mL binding buffer (20 mM Tris, pH 8.0, 50 mM KCl, 1 mM $MgCl_2$, 10% glycerol, 5 mM DTT) and 0-10,000 nM unlabeled T3 for 2 hours at 30°C. Protein-bound [^{125}I]T3 was captured by filtering through a nitrocellulose filter membrane (Millipore HA filters, 0.45 µm) under vacuum. The data was corrected for non-specific binding (counts bound at 10,000 nM unlabeled T3) and expressed as percentage maximal [^{125}I]T3 binding (counts bound at 0 nM unlabeled T3). The [^{125}I]T3 displacement curve and the dissociation constant (Kd) were computed by GraphPad Prism program version 5.0 (GraphPad, La Jolla, CA) and shown as mean ± standard error of the mean (SEM) of three independent experiments performed in duplicate.

Cell culture and transfection

JEG-3 cells were cultured and transfected as previously described (27). In brief, 20 ng of FLAG-tagged WT or mutant TRβ1 expression vectors and 120 ng of luciferase reporter constructs containing either direct repeat (DR4), inverted repeat (IR0) or everted repeat (ER6) thyroid hormone response element (TRE) (28), as well as 60 ng pMaxGFP transfection control, were transiently transfected into cells in TH depleted medium using Xtreme Gene 9 transfection reagent (Roche Diagnostics, Almere, The Netherlands). To determine the effect of TRβ1-L341V on WT function (dominant-negative effect), we co-expressed WT and TRβ1-L341V receptors (1:1 equimolar ratio), or either WT or TRβ1-L341V with empty vector (EV) (as gene dose control). After 24 hours, cells used for luciferase assays were incubated in DMEM/F12 medium supplemented with 0.1% bovine serum albumin and containing 0-10,000 nM T3 for 24 hours.

Immunoblotting

To determine the expression of FLAG-tagged TRβ1 WT and mutants in JEG-3 cells, nuclear proteins were extracted as described previously with slight modifications (29). Briefly, cells were swollen on ice for 15 min in buffer A (10 mM Hepes, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, pH 7.9) supplemented with the Complete Protease Inhibitor cocktail (Roche Diagnostics) and were lysed by addition of 0.6% NP40. The nuclei were pelleted by centrifugation for 10 min at 2500 *g* and extracted for 45 min in buffer C (20 mM HEPES, 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, Complete Protease Inhibitors, pH 7.9) at 4˚C. After centrifugation for 15 min at 20000 *g*, the supernatants containing nuclear proteins were collected and diluted in buffer D (20 mM HEPES, 1 mM EDTA, Complete Protease Inhibitors, pH 7.9). Immunoblotting was performed as previously described (27). The FLAG-tagged TRβ1 and Histone 3 (as loading control) were detected by FLAG-M2 antibody (#F1804 Sigma-Aldrich) and Histone 3 (H3; 1B1B2) antibody (#14269 Cell Signaling Technology), respectively, at a 1:1000 dilution and

visualized by Enhanced Chemiluminescence (Thermofisher Scientific) on the Alliance 4.0 Uvitec platform (Uvitec Ltd).

Luciferase assays

Luciferase activity of WT and mutant receptors was measured using the Dual Glo Luciferase kit (Promega, Leiden, The Netherlands) as previously described (22). The ratio between luciferase and GFP was calculated to adjust for transfection efficiency. Data were expressed as percentage maximal response of WT and half maximal effective T3 concentration (EC_{50}) and maximal response calculated using GraphPad Prism program version 5.0 (GraphPad, La Jolla, CA). The results are shown as mean ± SEM of at least three independent experiments performed in triplicate.

Statistical analysis

Statistical differences of logKd and logEC_{50} values between WT and mutants were analyzed by student's t-test. The percentage maximal response of mutants was compared to WT by one sample t-test. The statistical difference of logKd and logEC_{50} values between four mutants (L341V, L341A, L341I, and L341F) was determined by one-way ANOVA with Tukey's post-test. Statistical significance was considered when p-values < 0.05.

Results

Clinical and genetic assessment

A 12-year-old Thai girl (II.3) born to non-consanguineous parents presented with goiter and palpitations for four years. She had been diagnosed erroneously with Graves' disease and treated with methimazole for three years without remission. During treatment, she had fluctuating thyroid hormone and increased TSH concentrations. Physical examination showed a height of 134 cm (-3.17 SDS), a weight of 27.2 kg, a BMI of 15.1 kg/m^2 (-1.83 SDS), tachycardia (heart rate 144/min) and diffuse thyroid gland enlargement. Her thyroid function tests showed high FT4 and FT3 with non-suppressed TSH concentrations (Figure 1, Supplementary Table S2). Interestingly, an older sister (II.2) and her mother (I.2) also suffered from presumed Graves' disease, for which the mother had undergone a subtotal thyroidectomy and subsequently developed postoperative hypothyroidism, which required high doses of levothyroxine (300 µg/day). Because of the high TH with non-suppressed TSH concentrations of the index patient and affected family members, Graves' diagnosis was incorrect and RTHβ was suspected in this family.

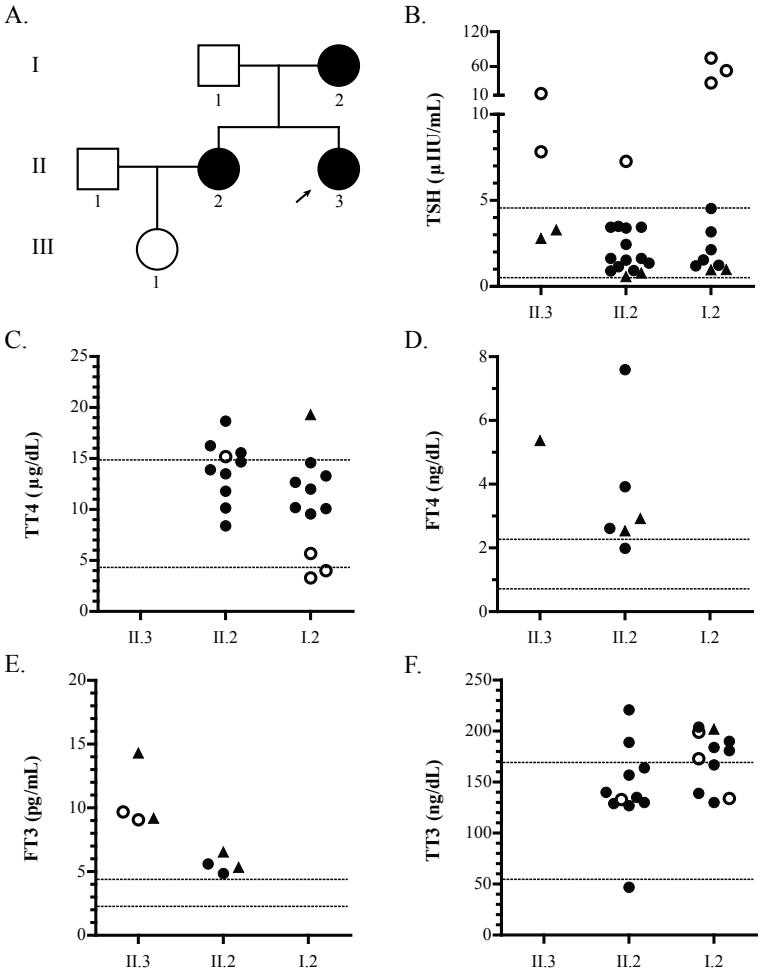


Figure 1. (A) The pedigree demonstrates three RTHβ patients in the family. (B-F) The graphs show thyroid function tests of affected family members (closed dot [●]: during antithyroid drug treatment and normal TSH; open dot [○]: during antithyroid drug treatment with high TSH; triangle [▲]: no treatment; [-----]: reference ranges).

After obtaining informed consent, genomic DNA of exons 7-10 of the *THRB* gene from the index patient and family members was sequenced and a novel heterozygous missense mutation at codon 341 leading to a leucine to valine substitution (c.1021C>G, p.L341V) was found in the index patient (II.3), the older sister (II.2) and the mother (I.2) (Supplementary Figure S1). This mutation was not found in the father (I.1) and is not present in public databases (dbSNP, 1000 Genomes, and Exome Aggregation Consortium [ExAC]).

In silico modeling of the TRβ1-L341V mutant

Inspection of the crystal structure of the T3-bound WT TRβ1 receptor (PDB-ID: 3GWS) revealed that Leu341 is located at the TRβ1 ligand-binding pocket (Figure 2A). Its aliphatic side-chain is predicted to form a direct hydrophobic interaction with the outer ring of the T3 molecule (Figure 2B). In addition, Leu341 interacts with several surrounding residues, amongst others the ligand-interacting residues Phe272, Leu330, and Leu346. In this way, Leu341 likely determines their orientation towards the T3 molecule and the overall shape and integrity of the ligand-binding pocket. Next, the L341V mutant was modeled into the T3-bound WT TRβ1 receptor crystal structure (Figure 4A). As a consequence of the shorter side-chain length of valine and an altered side-chain orientation as compared to the original leucine residue, it is not likely to form a direct interaction with ligand. Moreover, at least part of the hydrophobic interactions with surrounding residues, most importantly Leu330, is likely to be disturbed, which may impede the structural integrity of the ligand-binding pocket. All together, these observations suggest that the L341V mutant may reduce ligand-binding affinity and consequently receptor function.

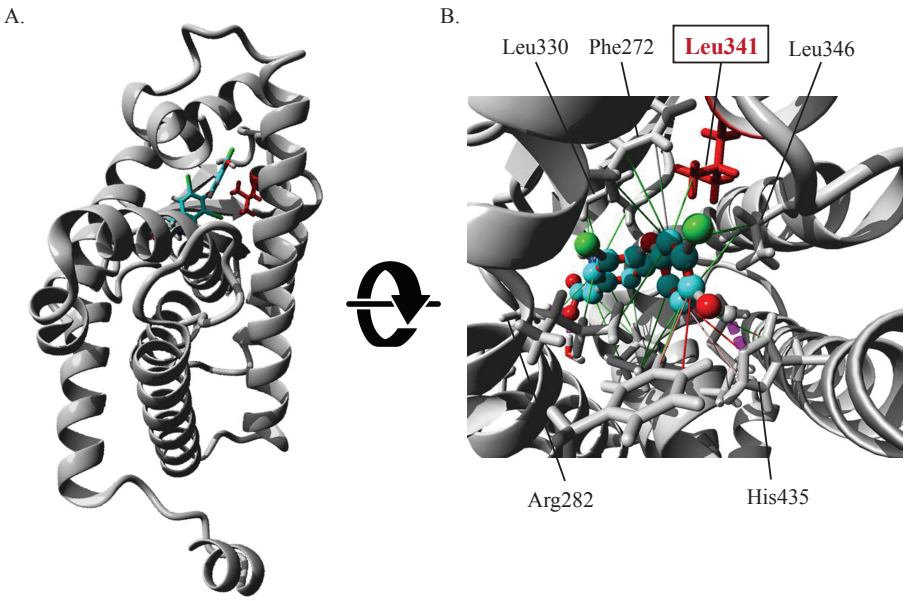


Figure 2. (A) Crystal structure of T3-bound WT TRβ1 (PDB-ID: 3GWS) in which the side chain of the affected Leu341 is depicted in red. (B) Close-up view of the ligand binding domain showing the side-chains of ligand-interacting residues. Arg282 and His435 form hydrogen bonds with the carboxyl group of the alanine side-chain and phenolhydroxyl group of T3, respectively (purple dashed lines). Together with Leu330, Phe272, and Leu346, Leu341 (in red) forms a hydrophobic pocket accommodating the two phenolic rings of the T3 molecule through hydrophobic (green lines) and pi-pi (pink-red lines) interactions.

In vitro functional analysis of TRβ1-L341V

To understand the effect of the L341V mutation on TRβ1 function, we performed [^{125}I]T3 competitive binding assays to determine the T3 binding affinity of the WT and mutant receptors. TRβ1-L341V showed a 16-fold higher dissociation constant (Kd) than WT TRβ1, indicating an impaired T3 binding affinity for the mutant (Figure 3A, Supplementary Table S3).

Next, we measured T3-dependent transcriptional activity of overexpressed WT or TRβ1-L341V receptors in JEG-3 cells using a luciferase reporter assay. The expression of receptor constructs was confirmed by immunoblotting (Figure 3B). The mutant receptor showed a significantly higher EC_{50} compared with WT TRβ1 on all TREs (60-fold on DR4, 40-fold on IR0, and 90-fold on ER6), reflecting an impaired T3-induced transcriptional activity of this mutation. However, the TRβ1-L341V showed similar maximal transcriptional activity with WT TRβ1, demonstrating that supra-physiological doses of T3 can rescue the transcriptional activity of the TRβ1-L341V (Figure 3C-E, Supplementary Table S3).

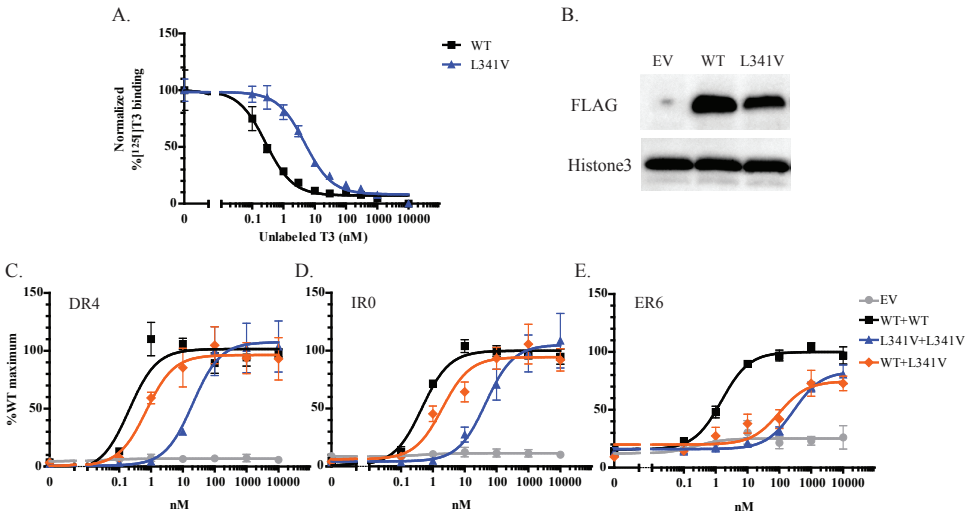


Figure 3. Functional analysis of TRβ1-L341V. (A) The [^{125}I]T3 dissociation curve of mutant shifts to the right suggesting an impaired affinity for T3 (data presented as mean ± SEM of three independent experiments performed in duplicate). (B) Immunoblotting confirms the expression of WT and TRβ1-L341V in JEG-3 cells. (C-E) The TRβ1-L341V shows impaired transcriptional activity on all TRE tested, as indicated by the right shift of the T3-induced dose-response curves. Co-transfection of WT with TRβ1-L341V alters transcriptional activity of WT in a dominant-negative manner (data presented as mean ± SEM of four independent experiments performed in triplicate).

The effect of TRβ1-L341V on WT TR function or its dominant-negative activity was tested by co-expressing WT and TRβ1-L341V. The EC_{50} of co-expressed WT and TRβ1-L341V was higher than that of WT only (3-fold on DR4, 5-fold on IR0, and 14-fold on ER6) suggesting a dominant-negative effect of the mutant receptor (Figure 3C-E, Supplementary Table S4). Together, these *in vitro* studies support an important role for Leu341 in substrate binding and receptor function.

In silico modeling and in vitro functional analysis of artificial mutations at 341 residue

To further delineate the function of Leu341, we generated expression constructs in which the Leu341 was substituted by an isoleucine (L341I), alanine (L341A), or phenylalanine (L341F). All of them have hydrophobic side-chains but of different sizes and structural properties (Figure 4A). In case of the isoleucine substitution, the branched chain character and the size of the side-chain are maintained, whereas the side-chain of an alanine is smaller. In contrast, the side-chain of phenylalanine is more bulky and rigid compared to the original isoleucine. Structural modeling of the L341I mutant suggested loss of direct contacts with ligand and slight alterations in the interactions with the surrounding residues. Obviously, these interactions were predicted to be even more extensively disturbed in case of the L341A mutant as a consequence of the size reduction of the side-chain. Although the bulky side-chain of phenylalanine was predicted to slightly alter the local architecture of the ligand-binding pocket, most of the essential interactions with the surrounding residues as well as the direct interaction(s) with the T3 molecule were preserved.

In vitro studies confirmed the functional impairment of these artificial mutants. The Kd of all three mutants indicating their affinity for T3, was significantly higher than WT. (Figure 4B, Supplementary Table S5). These results demonstrate that substitution of Leu341 by other non-polar amino acids with different side-chain size and orientation results in impaired T3 binding affinity of TRβ1. Interestingly, the shift in Kd was proportional to the size of the side-chain that was introduced and hence the distance to the substrate molecule and surrounding residues. Substitution by alanine and valine, which have a smaller size of the side-chain than isoleucine and phenylalanine, produced the higher shifts in Kd.

The shift of T3-induced transcriptional activity of the mutant receptors on the DR4 TRE showed a similar trend as the shift in affinity for T3. The EC_{50} of all mutants was significantly higher than that of WT TRβ1. In addition, the degree of the shift in EC_{50} also depended on the size and orientation of the side-chain (Figure 4C, Supplementary Table S5).

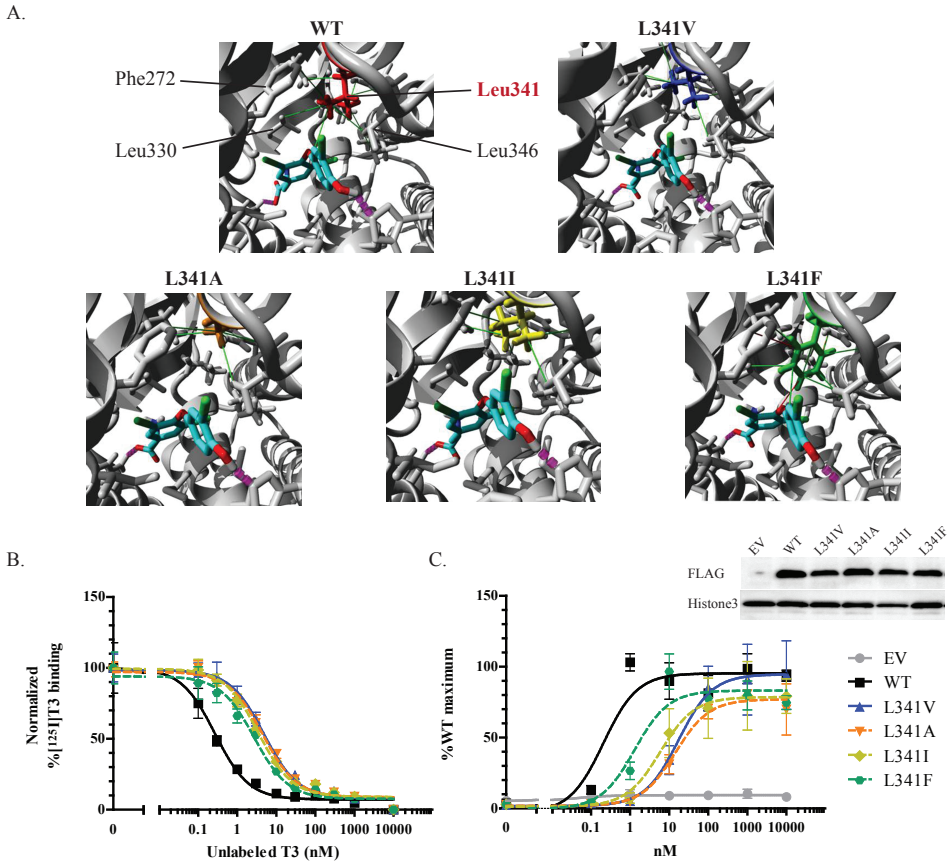


Figure 4. (A) Crystal structure of WT TRβ1 (PDB-ID: 3GWS) and structural models of the L341V and three artificial mutants (L341A, L341I and L341F) showing the side-chain size and orientation toward the T3 molecule and surrounding residues of the different residue side-chains. The WT Leu341 residue forms a direct interaction with ligand as well as extensive hydrophobic interactions (green lines) with its surrounding residues by which it stabilizes the hydrophobic pocket. All mutants were predicted to disturb these interactions to various degrees, with the L341F having the smallest impact. (B) The [^{125}I]T3 dissociation curve show the diverse severity of T3 binding impairment of the mutants (data presented as mean ± SEM of three independent experiments performed in duplicate). (C) Transcriptional activity of the mutant receptors is impaired, as indicated by the right-shifted of T3-induced dose-response curves tested on DR4 TRE (data presented as mean ± SEM of three independent experiments performed in triplicate). (Insert) Immunoblotting confirms the expression of all receptor constructs.

Discussion

In this study, we describe the role of Leu341 in TRβ function prompted by the identification of a novel TRβ1-L341V mutation in an RTHβ family. *In silico* modeling of TRβ1-L341V predicted interference with T3 binding, which was verified by [^{125}I]T3 competitive binding assays. Transcriptional impairment and dominant-negative ffect of this mutant was confirmed by *in vitro* studies. Additional artificial mutants (L341A, L341I, and L341F) were created based on the *in silico* predictions, showing different degrees of receptor impairment which depended on the side-chain size and exact orientation. With detrimental effects being observed even with subtle L341I and L341V mutations, illustrates the importance of Leu341 for TRβ1 function.

The novel L341V mutation reported in this study was identified in a patient and affected family members that had all been misdiagnosed with Graves' disease and treated with antithyroid drugs without remission. The mutation is located in a commonly mutated region (i.e., cluster 2) of TRβ1. Although the other mutation at this position, L341P, has been previously described (20,21), functional studies on the role of Leu341 in TRβ are lacking. Based on crystallographic analyses (24,30), Leu341 is predicted to be part of the TRβ1 ligand-binding surface.

Our *in silico* models suggest the presence of a direct hydrophobic interaction between Leu341 and the outer ring of the T3 molecule. Importantly, Leu341 also interacts with several other residues that line the ligand binding pocket, some of which (Phe272, Leu346) have been previously suggested to play an important role in ligand binding (15,31). Mutations in the latter have indeed been identified in RTHβ patients (20,32). Given its branched-chain character, Leu341 may therefore function as an important residue stabilizing the orientation of its surrounding residues. Shortening of its side-chain to valine would then affect the direct interaction with the T3 molecule, as well as the positioning and hence interactions of this residue with surrounding residues. The great impact of removing one side-chain methylene group in case of the TRβ1-L341V indeed suggests that the exact side-chain size and orientation at this position is of vital importance for substrate binding and hence receptor activity. We additionally confirmed this by studying three artificial mutations, L341A, L341I and L341F. The side-chains of these three amino acids all have similar hydrophobic properties but a different size and orientation, resulting in a variable distance to the T3 molecule.

The *in vitro* studies confirmed the results of the *in silico* modeling. The TRβ1-L341V has a reduced affinity for T3, as indicated by the higher Kd compared to WT TRβ1, and impaired T3-dependent transcriptional activity, as indicated by the increased EC_{50} on all TREs tested. These data illustrate the resistance of TRβ1-L341V to T3 stimulation, which can, however, be overcome in case of higher (supra-physiological) doses of T3. Such rescue of function at high T3 concentrations has also been described with other TRβ1 mutations (33-35) and is probably due to the fact that T3 binding affinity of these mutants is not completely

abolished. In addition to the *in vitro* studies of TRβ1-L341V, the three artificial mutants also showed significantly reduced affinity for T3 and impaired transcriptional activity. Interestingly, the degree of the shift in EC_{50} related to the distance between the side-chain and T3. These results further confirm that the interaction between T3 and TRβ1 receptor requires a proper distance between the amino acid at position 341 and inner ring iodine of T3.

TRβ1-L341V also showed a dominant-negative effect on transcriptional activity of co-expressed WT TRβ1 on all TREs tested, but strongest on the ER6 TRE. The strong dominant-negative effect on this TRE has also been observed in other TRβ1 mutants (34,36). In contrast to the DR4 and IR0 TREs, where TRβ1 engages predominantly as a heterodimer with RXR, TRβ1 acts on ER6 as a homodimer (37). This means that in the heterozygous situation, only 25% of homodimers are formed by WT receptors. If both receptors of the homodimer need to bind ligand in order to exert transcriptional activity, this may explain the stronger dominant-negative effects on ER6 TRE.

To our knowledge, this is the first study showing the role of the Leu341 residue in TRβ function, gaining more detailed insight into T3 and TRβ interaction. Taking into account the predictive nature of structural modelling, we managed to develop an *in silico* model that correctly predicts the degree of receptor impairment compared with *in vitro* studies. In addition, the creation of artificial mutations based on the *in silico* modeling proved to be a good approach to further explore the role and importance of affected amino acid residues.

In conclusion, we verify the importance of the Leu341 residue for TRβ function. The interactions between Leu341, its surrounding residues, and T3 are required for optimal affinity for T3 and transcriptional activity. Mutations at this amino acid residue can reduce affinity for T3 and impair transcriptional activity of TRβ.

Acknowledgements

We thank Dr. Dounghatai Pongpanat for her contribution to provide patients' information and help to contact family members. This work is supported by Zon-MWTOP Grant (91212044), an Erasmus MC Medical Research Advisory Committee (MRACE) grant (R.P.P., M.E.M.), Chiang Mai University (K.W.), and NIHR Cambridge Biomedical Centre (V.K.C.).

Author Disclosure Statement

The authors have nothing to disclose.

References

1. Cheng SY, Leonard JL, Davis PJ. Molecular aspects of thyroid hormone actions. *Endocr Rev.* 2010;**31**(2):139-170.
2. Refetoff S, DeWind LT, DeGroot LJ. Familial syndrome combining deaf-mutism, stuppled epiphyses, goiter and abnormally high PBI: possible target organ refractoriness to thyroid hormone. *J Clin Endocrinol Metab.* 1967;**27**(2):279-294.
3. Sakurai A, Takeda K, Ain K, Ceccarelli P, Nakai A, Seino S, Bell GI, Refetoff S, DeGroot LJ. Generalized resistance to thyroid hormone associated with a mutation in the ligand-binding domain of the human thyroid hormone receptor beta. *Proc Natl Acad Sci U S A.* 1989;**86**(22):8977-8981.
4. Lafranchi SH, Snyder DB, Sesser DE, Skeels MR, Singh N, Brent GA, Nelson JC. Follow-up of newborns with elevated screening T4 concentrations. *J Pediatr.* 2003;**143**(3):296-301.
5. Tajima T, Jo W, Fujikura K, Fukushi M, Fujieda K. Elevated free thyroxine levels detected by a neonatal screening system. *Pediatr Res.* 2009;**66**(3):312-316.
6. Dumitrescu AM, Refetoff S. The syndromes of reduced sensitivity to thyroid hormone. *Biochim Biophys Acta.* 2013;**1830**(7):3987-4003.
7. Singh BK, Yen PM. A clinician's guide to understanding resistance to thyroid hormone due to receptor mutations in the TRalpha and TRbeta isoforms. *Clin Diabetes Endocrinol.* 2017;**3**:8.
8. Maruo Y, Mori A, Morioka Y, Sawai C, Mimura Y, Matui K, Takeuchi Y. Successful every-other-day liothyronine therapy for severe resistance to thyroid hormone beta with a novel THRB mutation; case report. *BMC Endocr Disord.* 2016;**16**:1.
9. Phillips SA, Rotman-Pikielny P, Lazar J, Ando S, Hauser P, Skarulis MC, Brucker-Davis F, Yen PM. Extreme thyroid hormone resistance in a patient with a novel truncated TR mutant. *J Clin Endocrinol Metab.* 2001;**86**(11):5142-5147.
10. Miyoshi Y, Nakamura H, Tagami T, Sasaki S, Dorin RI, Taniyama M, Nakao K. Comparison of the functional properties of three different truncated thyroid hormone receptors identified in subjects with resistance to thyroid hormone. *Mol Cell Endocrinol.* 1998;**137**(2):169-176.
11. Ribeiro RC, Apriletti JW, Wagner RL, Feng W, Kushner PJ, Nilsson S, Scanlan TS, West BL, Fletterick RJ, Baxter JD. X-ray crystallographic and functional studies of thyroid hormone receptor. *J Steroid Biochem Mol Biol.* 1998;**65**(1-6):133-141.
12. Ye L, Li YL, Mellstrom K, Mellin C, Bladh LG, Koehler K, Garg N, Garcia Collazo AM, Litten C, Husman B, Persson K, Ljunggren J, Grover G, Sleph PG, George R, Malm J. Thyroid receptor ligands. 1. Agonist ligands selective for the thyroid receptor beta1. *J Med Chem.* 2003;**46**(9):1580-1588.
13. Wagner RL, Huber BR, Shiau AK, Kelly A, Cunha Lima ST, Scanlan TS, Apriletti JW, Baxter JD, West BL, Fletterick RJ. Hormone selectivity in thyroid hormone receptors. *Mol Endocrinol.* 2001;**15**(3):398-410.
14. Bleicher L, Aparicio R, Nunes FM, Martinez L, Gomes Dias SM, Figueira AC, Santos MA, Venturelli WH, da Silva R, Donate PM, Neves FA, Simeoni LA, Baxter JD, Webb P, Skaf MS, Polikarpov I. Structural basis of GC-1 selectivity for thyroid hormone receptor isoforms. *BMC Struct Biol.* 2008;**8**:8.
15. Martinez L, Nascimento AS, Nunes FM, Phillips K, Aparicio R, Dias SM, Figueira AC, Lin JH, Nguyen P, Apriletti JW, Neves FA, Baxter JD, Webb P, Skaf MS, Polikarpov I. Gaining ligand selectivity in thyroid hormone receptors via entropy. *Proc Natl Acad Sci U S A.* 2009;**106**(49):20717-20722.
16. Nomura Y, Nagaya T, Tsukaguchi H, Takamatsu J, Seo H. Amino acid substitutions of thyroid hormone receptor-beta at codon 435 with resistance to thyroid hormone selectively alter homodimer formation. *Endocrinology.* 1996;**137**(10):4082-4086.
17. Collingwood TN, Wagner R, Matthews CH, Clifton-Bligh RJ, Gurnell M, Rajanayagam O, Agostini M, Fletterick RJ, Beck-Peccoz P, Reinhardt W, Binder G, Ranke MB, Hermus A, Hesch RD, Lazarus J, Newrick P, Parfitt V, Raggatt P, de Zegher F, Chatterjee VK. A role for helix 3 of

the TRbeta ligand-binding domain in coactivator recruitment identified by characterization of a third cluster of mutations in resistance to thyroid hormone. *EMBO J.* 1998;**17**(16):4760-4770.

18. Li F, Xie Q, Li X, Li N, Chi P, Chen J, Wang Z, Hao C. Hormone activity of hydroxylated polybrominated diphenyl ethers on human thyroid receptor-beta: in vitro and in silico investigations. *Environ Health Perspect.* 2010;**118**(5):602-606.
19. Hangeland JJ, Friends TJ, Doweyko AM, Mellstrom K, Sandberg J, Grynfarb M, Ryono DE. A new class of high affinity thyromimetics containing a phenyl-naphthylene core. *Bioorg Med Chem Lett.* 2005;**15**(20):4579-4584.
20. Rivolta CM, Olcese MC, Belforte FS, Chiesa A, Gruneiro-Papendieck L, Iorcansky S, Herzovich V, Cassorla F, Gauna A, Gonzalez-Sarmiento R, Targovnik HM. Genotyping of resistance to thyroid hormone in South American population. Identification of seven novel missense mutations in the human thyroid hormone receptor beta gene. *Mol Cell Probes.* 2009;**23**(3-4):148-153.
21. Chiesa A, Olcese MC, Papendieck P, Martinez A, Vieites A, Bengolea S, Targovnik HM, Rivolta CM, Gruneiro-Papendieck L. Variable clinical presentation and outcome in pediatric patients with resistance to thyroid hormone (RTH). *Endocrine.* 2012;**41**(1):130-137.
22. van Mullem A, van Heerebeek R, Chrysis D, Visser E, Medici M, Andrikoula M, Tsatsoulis A, Peeters R, Visser TJ. Clinical phenotype and mutant TRalpha1. *N Engl J Med.* 2012;**366**(15):1451-1453.
23. Krieger E, Vriend G. YASARA View - molecular graphics for all devices - from smartphones to workstations. *Bioinformatics.* 2014;**30**(20):2981-2982.
24. Nascimento AS, Dias SM, Nunes FM, Aparicio R, Ambrosio AL, Bleicher L, Figueira AC, Santos MA, de Oliveira Neto M, Fischer H, Togashi M, Craievich AF, Garratt RC, Baxter JD, Webb P, Polikarpov I. Structural rearrangements in the thyroid hormone receptor hinge domain and their putative role in the receptor function. *J Mol Biol.* 2006;**360**(3):586-598.
25. Canutescu AA, Shelenkov AA, Dunbrack RL, Jr. A graph-theory algorithm for rapid protein side-chain prediction. *Protein Sci.* 2003;**12**(9):2001-2014.
26. Krieger E, Joo K, Lee J, Lee J, Raman S, Thompson J, Tyka M, Baker D, Karplus K. Improving physical realism, stereochemistry, and side-chain accuracy in homology modeling: Four approaches that performed well in CASP8. *Proteins.* 2009;**77 Suppl 9**:114-122.
27. van Gucht AL, Meima ME, Zwaveling-Soonawala N, Visser WE, Fliers E, Wennink JM, Henny C, Visser TJ, Peeters RP, van Trotsenburg AS. Resistance to Thyroid Hormone Alpha in an 18-Month-Old Girl: Clinical, Therapeutic, and Molecular Characteristics. *Thyroid.* 2016;**26**(3):338-346.
28. Collingwood TN, Adams M, Tone Y, Chatterjee VK. Spectrum of transcriptional, dimerization, and dominant negative properties of twenty different mutant thyroid hormone beta-receptors in thyroid hormone resistance syndrome. *Mol Endocrinol.* 1994;**8**(9):1262-1277.
29. Fozzatti L, Lu C, Kim DW, Cheng SY. Differential recruitment of nuclear coregulators directs the isoform-dependent action of mutant thyroid hormone receptors. *Mol Endocrinol.* 2011;**25**(6):908-921.
30. Wagner RL, Apriletti JW, McGrath ME, West BL, Baxter JD, Fletterick RJ. A structural role for hormone in the thyroid hormone receptor. *Nature.* 1995;**378**(6558):690-697.
31. Borngraeber S, Budny MJ, Chiellini G, Cunha-Lima ST, Togashi M, Webb P, Baxter JD, Scanlan TS, Fletterick RJ. Ligand selectivity by seeking hydrophobicity in thyroid hormone receptor. *Proc Natl Acad Sci U S A.* 2003;**100**(26):15358-15363.
32. Macchia E, Gurnell M, Agostini M, Giorgilli G, Marcocci C, Valenti TM, Martino E, Chatterjee KK, Pinchera A. Identification and characterization of a novel de novo mutation (L346V) in the thyroid hormone receptor beta gene in a family with generalized thyroid hormone resistance. *Eur J Endocrinol.* 1997;**137**(4):370-376.
33. Maraninchi M, Bourcigaux N, Dace A, El-Yazidi C, Malezet-Desmoulins C, Krempf M, Torresani J, Margotat A. A novel mutation (E333D) in the thyroid hormone beta receptor causing resistance to thyroid hormone syndrome. *Exp Clin Endocrinol Diabetes.* 2006;**114**(10):569-576.
34. Zavacki AM, Harney JW, Brent GA, Larsen PR. Dominant negative inhibition by mutant thyroid

hormone receptors is thyroid hormone response element and receptor isoform specific. *Mol Endocrinol.* 1993;**7**(10):1319-1330.

35. Chatterjee VK, Nagaya T, Madison LD, Datta S, Rentoumis A, Jameson JL. Thyroid hormone resistance syndrome. Inhibition of normal receptor function by mutant thyroid hormone receptors. *J Clin Invest.* 1991;**87**(6):1977-1984.

36. Zavacki AM, Harney JW, Brent GA, Larsen PR. Structural features of thyroid hormone response elements that increase susceptibility to inhibition by an RTH mutant thyroid hormone receptor. *Endocrinology.* 1996;**137**(7):2833-2841.

37. Chen Y, Young MA. Structure of a thyroid hormone receptor DNA-binding domain homodimer bound to an inverted palindrome DNA response element. *Mol Endocrinol.* 2010;**24**(8):1650-1664.

Supplementary Materials

Supplementary Table S1. Primers for *THRB* sequencing, FLAG-TRβ1 cloning, and FLAG-TRβ1 mutagenesis.

Condition	Primers (5'-3')
Exon 7 *THRA*	Forward: TGCAGCTTGCTGTGTATCTTG Reverse: CCCAAGGTGATGAGGACTG
Exon 8 *THRA*	Forward: CTTTCTGCAGCAACAGTCC Reverse: GTATTCCTGGAAACTGATGAAAC
Exon 9 *THRA*	Forward: GAAAACCATGGGCTCAAAG Reverse: TGAAGCTAAAGGGGGACTG
Exon 10 *THRA*	Forward: TAAAGGCCTGGAATTGGAC Reverse: TGCTTGGTGCTGGTGAG
FLAG-TRβ1 cloning	Forward: GTAGAATTCTGGCCGCAGAAATGGACTACAAAGACGATGACG ACAAGATGACTCCCAACAGTATGACAGAAAATG Reverse: CTATCTAGACTAATCCTCGAACACTTCCAAGAAC
L341V (CTG>GTG)	Forward: GACACGGGGCCAGGTGAAAAATGGGGG Reverse: CCCCCATTTTTCACCTGGCCCCGTGTC
L341A (CTG>GCG)	Forward: GTGACACGGGGCCAGGCGAAAAATGGGGGTCT Reverse: AGACCCCCATTTTTCGCCTGGCCCCGTGTCAC
L341I (CTG>ATC)	Forward: AGTGACACGGGGCCAGATCAAAAATGGGGGTCTTG Reverse: CAAGACCCCCATTTTTGATCTGGCCCCGTGTCACT
L341F (CTG>TTC)	Forward: AGTGACACGGGGCCAGTTCAAAAATGGGGGTCTTG Reverse: CAAGACCCCCATTTTTGAACTGGCCCCGTGTCACT

Supplementary Table S2. Clinical and biochemical characteristics of index patient and family members.

Parameter	Index case (II.3)	Sister (II.2)	Mother (I.2)
Age (year)	12	23	51
Clinical presentation	goiter, tachycardia	goiter, tachycardia	goiter
Thyroid function tests [normal range]			
- TSH (μIU/mL) [0.5-4.8]	3.29	2.45	1.2
- TT4 (μg/dL) [4.2-13.0]	N/A	13.5	12
- FT4 (ng/dL) [0.8-2.3]	5.37	N/A	N/A
- TT3 (ng/dL) [55-170]	N/A	221	190
- FT3 (pg/mL) [2.3-4.2]	14.31	N/A	N/A
- Anti-TPO (IU/mL) [<40]	5.0	N/A	N/A
- Anti-TG (IU/mL) [<125]	19.9	N/A	N/A
- TRAb (IU/L) [0.00-1.75]	<0.3	<0.3	N/A
Previous treatment	MMI 15 mg/day	MMI	MMI then subtotal thyroidectomy

TSH, thyroid-stimulating hormone; TT4, total thyroxine; FT4, free thyroxine; TT3, total triiodothyronine; FT3, free triiodothyronine; Anti-TPO, anti-thyroid peroxidase; Anti-TG, anti-thyroglobulin; TRAb, thyrotropin receptor autoantibody; MMI, methimazole; N/A, not available

Supplementary Table S3. Functional analysis of WT and TRβ1-L341V.

Parameter	TRE	WT TRβ1	TRβ1-L341V
LogKd [Kd(nM)]	-	-0.51±0.13 [0.31]	0.70±0.06** [5.01]
LogEC50 [EC_{50}(nM)]	DR4	-0.45±0.04 [0.35]	1.32±0.04*** [21.1]
	IR0	-0.17±0.04 [0.67]	1.69±0.16*** [49.5]
	ER6	0.31±0.09 [2.03]	2.43±0.16*** [269]
% WT maximal response	DR4	100	106±22.6
	IR0	100	107±22.5
	ER6	100	83.9±8.41

student's t-test compared to WT, *p<0.05, **p<0.01, ***p<0.001

Supplementary Table S4. The dominant-negative effect of TRβ1-L341V on transcriptional activity of WT receptor.

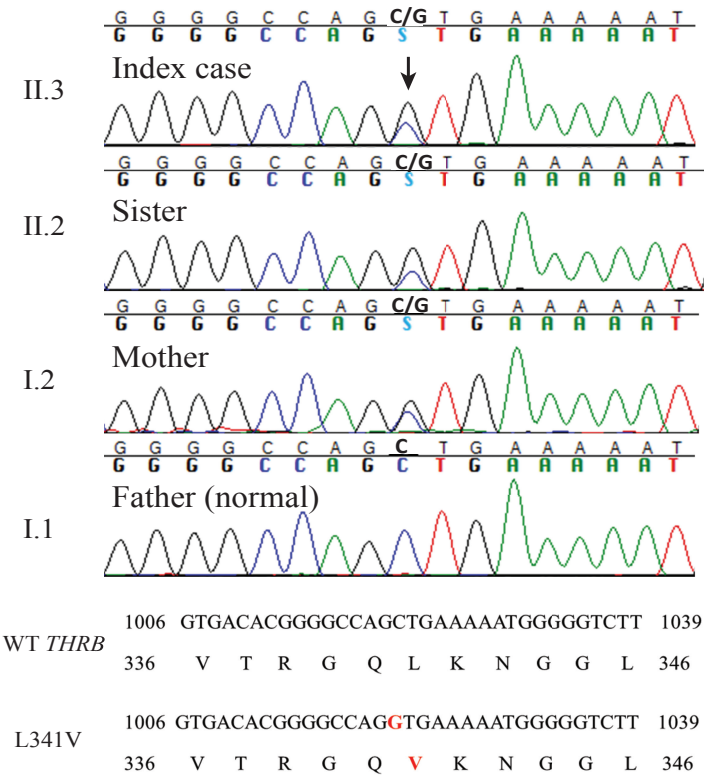
Parameter	TRE	WT+WT	WT+EV	L341V+ L341V	L341V+EV	WT+L341V	p-value (One way ANOVA)
$LogEC_{50}$ $[EC_{50}(nM)]$	DR4	-0.45±0.04 [0.35]	-0.47±0.04 [0.34]	1.32±0.04 [21.1]	1.36±0.02 [22.9]	$0.05±0.13^{***, ttt}$ [1.11]	<0.0001
	IR0	-0.17±0.04 [0.67]	-0.11±0.15 [0.77]	1.69±0.16 [49.5]	1.53±0.18 [33.7]	$0.52±0.13^{*,t}$ [3.29]	<0.0001
	ER6	0.31±0.09 [2.03]	0.32±0.30 [2.11]	2.43±0.16 [269]	2.43±0.19 [267]	1.46±0.45 [28.9]	<0.0001

Tukey's post-test compared to WT+WT, *p<0.05, **p<0.01, ***p<0.001, and WT+EV, †p<0.05, ††p<0.01, †††p<0.001

Supplementary Table S5. Functional analysis of artificial mutants at 341 amino acid residue.

Parameter	WT TRβ1	TRβ1-L341V	TRβ1-L341A	TRβ1-L341I	TRβ1-L341F	p-value (One way ANOVA)
LogKd [Kd(nM)]	-0.51±0.13 [0.31]	$0.70±0.06^{**}$ [5.01]	$0.66±0.06^{**}$ [4.54]	$0.47±0.10^{**}$ [2.94]	$0.53±0.11^{**}$ [3.42]	0.2606
$LogEC_{50}$ $[EC_{50}(nM)]$	-0.47±0.03 [0.34]	$1.33±0.04^{***}$ [21.38]	$1.37±0.04^{***}$ [23.12]	$1.19±0.35^{***}$ [15.35]	$0.33±0.06^{***}$ [2.14]	0.0113

student's t-test compared to WT, **p<0.01, ***p<0.001



Supplementary Figure S1 Sequence analysis of exon 9 of *THRB* shows a heterozygous missense mutation (c.1021C>G) in index case (II.3), older sister (II.2) and mother (I.2), resulting in a valine substitution at codon 341 (p.L341V).