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Anomalous Behavior of Disease-Inflicting Polymorphic Variants of Nuclear Receptor THRβ of Indian Origin

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Abstract

Thyroid hormone receptor β (THRβ) binds to thyroid hormones to execute various cellular and physiological processes as a ligand-inducible transcriptional factor. THRs, especially THRβ, are key players in the central regulation of the HPT axis. They ensure a delicate balance between thyroid hormone production and feedback control, allowing the body to adapt to changing environmental conditions. Polymorphisms in THRβ can lead to multiple clinical manifestations like resistance to thyroid hormone β, neurological or psychological disorders (like autism, intellectual disabilities, etc.), and several types of cancers (papillary thyroid cancer, breast cancer, etc.). This study examined two disease-inflicting polymorphic variants of THRβ, P323L, and P453S of Indian origin. It was observed that these variants exhibit impaired subcellular localization patterns, transcriptional functions, and compromised receptor stability. The study provides valuable insight into the cellular mechanism underlying receptor dysfunction and inflicting disease states. It is anticipated that disease-inflicting polymorphic variants of THRβ influence the structural and functional behaviour of the receptor, contributing to the onset of disease. A concerted effort to gain the molecular basis of receptor dysregulation will help improve the assessment and management of THRβ-mediated diseases.

Keywords: Autism Spectrum Disorder, Polymorphism, Resistance to Thyroid Hormone, Thyroid Hormone, Thyroid Hormone Receptor Beta

Abbreviations: **NR:** Nuclear receptor, **THRβ:** Thyroid hormone receptor beta, **TH:** Thyroid hormone, **T_3:** 3,3′,5-triiodothyronine, **TRE:** Thyroid hormone response element, **RTH:** Resistance to thyroid hormone

1. Introduction

The thyroid gland regulates most of the metabolic and physiological processes of the body. Thyroid hormones (THs), secreted from the thyroid gland, govern diverse physiological functions and gene expression[1,2]. Two predominant endogenous THs produced by the thyroid gland are 3,3′,5-triiodothyronine (T_3), and thyroxine (T_4). The thyroid hormone receptors (THRs) are classical nuclear hormone receptors that function as T_3-inducible transcriptional regulators and bind to specific response elements on the promoters of TH-responsive genes[1-3]. Through binding to the active form of TH (T_3), THR transcriptionally regulates various metabolic processes, liver functions, heart rate, and muscle and bone functions[3].

THRα and THRβ are the product of two distinct genes (*THRA* and *THRB*) located on different chromosomes. THRα1 and THRβ1 isoforms are ubiquitously expressed in the human body. THRα1 is found in the bone, myocardial and skeletal muscles, central nervous, and gastrointestinal systems. THRβ1 is abundantly expressed in the liver and kidney, developing nervous system, spleen, pituitary, and hypothalamus, and THRβ2 is predominant in the pituitary, hypothalamus, inner ear, and retina[2,4,5]. Aberrations or malfunctioning in THRβ function develops into thyroid dysfunction, and polymorphism in the *THRB* gene has been associated with several diseases, including resistance to thyroid hormone β (RTHβ), malignancies, psychological or neurological disorders, etc.[6-10].

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Thyroid diseases affect 42 million Indians, with the majority of them being females with hypothyroidism[11]. When circulating levels of thyroid hormones (T_3 and T_4) are optimal, they bind to THRβ in the hypothalamus and pituitary, leading to the inhibition of TRH and TSH production, respectively. This negative feedback loop helps maintain thyroid hormone homeostasis[12-14]. Over 230 heterozygous pathogenic THRβ variants have been reported and are predominantly missense[5]. Two THRβ variants in Indian populations are observed to be significantly associated with two major diseases, i.e., RTHβ and autism spectrum disorder (ASD). The defining features of RTHβ are elevated THs (T_4 and T_3) levels with normal or slightly increased TSH levels[5]. A case report, identified for the first time in 2017, has highlighted the association of a novel mutation (P453S) from the hot spot region of THRβ with RTHβ[6]. Furthermore, clinical research has established a correlation between the thyroid hormone signalling pathway and neuron development[7]. Another Indian study has identified a P323L-THRb polymorphism that is associated with ASD.[7]. However, the molecular basis of disease genesis associated with these THRβ variants remains elusive.

The two disease-inflicting THRβ variants have been investigated only clinically and await further investigation for functional and structural aberrations. The present study aimed to investigate the anomalous behaviour of these two disease-causing THRβ polymorphic variants (P323L and P453S) found in the Indian population. In view of this, we have examined these variants using *in silico*, molecular, and cellular approaches. Our study reveals that these disease-associated THRβ variants exhibit deviant receptor localization and altered transcriptional response. The above-observed alterations in the functioning of THRβ variants, derived from different test parameters, provided insights into the onset of thyroid-related disorders. Mechanistic understanding of these polymorphic variants may facilitate the development of genomic-based diagnosis, leading to better predictions and subsequent disease management.

2. Materials and Methods

2.1 Plasmid Constructs and Reagents

Wild-type RFP-tagged THRβ expression plasmid was generated using pRSV-hTHRβ as a template, which was a kind gift from Dr. J. Weitzel (Institute of Reproductive Biology, Leibniz Institute for Farm Animal Biology, Germany)[15,16]. THRβ polymorphic variants (P323L and P453S) were generated using specific primers, wild-type RFP-tagged THRβ as a template and employing a site-directed mutagenesis approach (Q5® Site-directed mutagenesis kit, New England Biolabs, USA). Mutations introduced were confirmed by sequencing. DR-4 Luc or TRE-Luc (TH response element with reporter firefly luciferase gene) was a gift from Dr. M. Figueira[17]. GFP-RXR has been reported earlier[18]. T_3 hormone or 3,3′,5-Triiodo-L-thyronine (T2877) was purchased from Sigma-Aldrich (St. Louis, MO, USA). β-Galactosidase (β-gal) was from Invitrogen, #V800-20. Cell culture reagents, including Dulbecco's Modified Eagle's Medium (DMEM) and Fetal Bovine Serum (FBS), were procured from Sigma-Aldrich (St. Louis, MO, USA) and Gibco Inc. (USA), respectively. The anti-mycotic solution was from Himedia (India). Transfection reagent polyethyleneimine (cationic polymer PEI linear) was provided by Polysciences, Inc. Plasticware for mammalian cell culture experiments were purchased from Corning Costar Corp. (Lowell, MA, USA). The remaining general chemicals and reagents were of analytical grade and procured from various commercial suppliers.

2.2 *In Silico* Receptor Analysis with SIFT, PolyPhen 2.0, and I-Mutant Tools

The protein sequence of THRβ, obtained from Uniprot (ID: P10828) in FASTA format, was analyzed using three computational tools to predict the effect of polymorphism. These tools are SIFT, PolyPhen 2.0, and I-Mutant. Each tool provides information on different aspects of the polymorphism. SIFT, an online software, assesses the functional impact of missense mutations through sequence homology analysis (https://sift.bii.a-star.edu.sg/). It predicts missense mutation as deleterious (score<0.05) or tolerated (score≥0.05)[19,20]. PolyPhen2.0 is an online tool to predict the functional effects of amino acid substitutions on human proteins (http://genetics.bwh.harvard.edu/pph2/index.shtml). Based on the score, mutants are classified as benign (score≤0.5), possibly damaging (0.5<score ≤0.9), or probably damaging (score>0.9)[20,21]. The I-Mutant suite uses support vector machines to predict variations in protein stability caused by single-point substitutions (https://folding.biofold.org ›i-mutant›i-mutant2.0.html). It analyses ΔΔG after the mutation. A negative ΔΔG value indicates low stability of the protein, and vice versa.[22].

2.3 Maintenance of Cell Culture

HEK-293T (human embryonic kidney cell line) cells were procured from the National Centre for Cell

Science(NCCS), Pune, India. HEK-293T cells were routinely cultured and maintained in complete DMEM media containing 10% FBS with antibiotic and anti-mycotic components (0.25 µg/mL amphotericin, 100 µg/mL streptomycin, and 100 µg/mL of penicillin) at 95% atmospheric air and 5% CO_2 at a temperature of 37^{0}C.

2.4 Transient Transfection and Live-Cell Imaging

The cells were seeded in complete DMEM in 35 mm sterile culture plates. After 24 h, when cells reached a confluency of ~60%, the transfection was performed using PEI and plasmid in a 3:1 ratio. After 4 h of the transfection period, the cells were supplemented with steroid-stripped DMEM media. The cells were transfected with either wild-type or THRβ variants or with heterodimeric partner RXR as per experimental requirements. After 4 h, the cells were treated either with solvent (DMSO: Ethanol) or 100 nM T_3 hormone for nucleus staining. Hoechst was added at least 2 h prior to imaging performed under the fluorescence microscope with water immersion objectives (Evolution VF, Media Cybernetics, USA). For every replicate, 100 cells were counted and categorized into five categories based on fluorescence distribution. The study used a five-category classification method to determine the subcellular localization of receptor[23]. The method involved identifying fluorescence in the nuclear compartment (N), predominantly nuclear (N>C), equal amounts of fluorescence in the cytoplasm and nucleus (N=C), mostly cytoplasmic localized (C>N), and fluorescence entirely confined to the cytoplasm (C) (Figure 2A).

2.5 Promoter-Reporter-based Luciferase Assay

In 12-well tissue culture plates, HEK-293T cells were seeded one day before transfection in complete DMEM media. At approximately 60% confluency, the cells were transfected with different THRβ constructs and TRE-Luc along with β-gal plasmid in Opti-MEM media. After the transfection period, the cells were supplemented with charcoal-stripped serum media and treated with either solvent or T_3 (100 nM). After 24 h of hormone treatment, cells were washed with PBS, lysed, and extracted in reporter lysis buffer. The reporter luciferase activity was measured using the manufacturer's protocol (Promega, Madison, WI, USA). β-Galactosidase activity from the extracts was used to normalize the efficiency of transfection.

2.6 Statistical Analysis

All experiments were repeated at least three times, with values representing the mean ± SE of three independent experiments. The student's t-test was applied to compare the values for significance, with asterisks (*) indicating significant differences from control experiments with p-values < 0.05.

3. Results

3.1 Comparative *In Silico* Analysis of Disease-Associated THRβ Polymorphic Variants using PolyPhen 2.0, SIFT, and I-Mutant

SIFT, PolyPhen 2.0, and I-Mutant *in silico* prediction tools were used to predict the impact of non-synonymous substitutions on protein stability and their pathogenic potential. The SIFT, a sequence homology-based tool, was used to assess the conservation of a protein's specific amino acid residue position. The SIFT results demonstrated that substituting a proline residue at 323 and 453 positions in THRβ protein was deleterious. Additionally, to analyze the structural and functional effect of the substitution of amino acids, PolyPhen version 2.0 was used. The THRβ variants, P323L and P453S, were found to be damaging upon PolyPhen analysis. Furthermore, to predict the changes in the protein stability caused by single-point polymorphism, the I-Mutant tool was utilized. Both THRβ variants resulted in decreased protein structure stability with negative ΔΔG values as analyzed by I-Mutant. The detailed computational analysis of both variants according to three tools has been summarised in Table 1.

3.2 Disease-Associated THRβ Variants Exhibit Altered Subcellular Localization

To study the effect of polymorphism on the subcellular localization pattern of THRβ, we generated the two variants and confirmed by sequencing method. Further, to perform live-cell imaging experiments, we transiently transfected HEK-293T cells with RFP-tagged expression constructs, i.e., wild-type THRβ and its variants. The localization pattern of disease-associated THRβ variants was determined by analyzing the fluorescence intensity in living cells and categorized based on the five-category classification described in 'Material and Methods' and presented in Figure 1A. The localization of unliganded

Tabe 1. *In silico* analysis of disease-associated THRβ variants by three computational programs: SIFT, PolyPhen 2.0, and I-Mutant 3.0

Amino acid change	SIFT		PolyPhen 2.0		I-Mutant	
	Tolerance Index	Prediction	Prediction	Score	ΔΔG (Kcal/mol)	Stability
P323L (ASD)	0.02	Deleterious	Possibly damaging	0.895	Decrease	-0.72
P453S (RTH)	0.00	Deleterious	Probably damaging	1.000	Decrease	-1.16

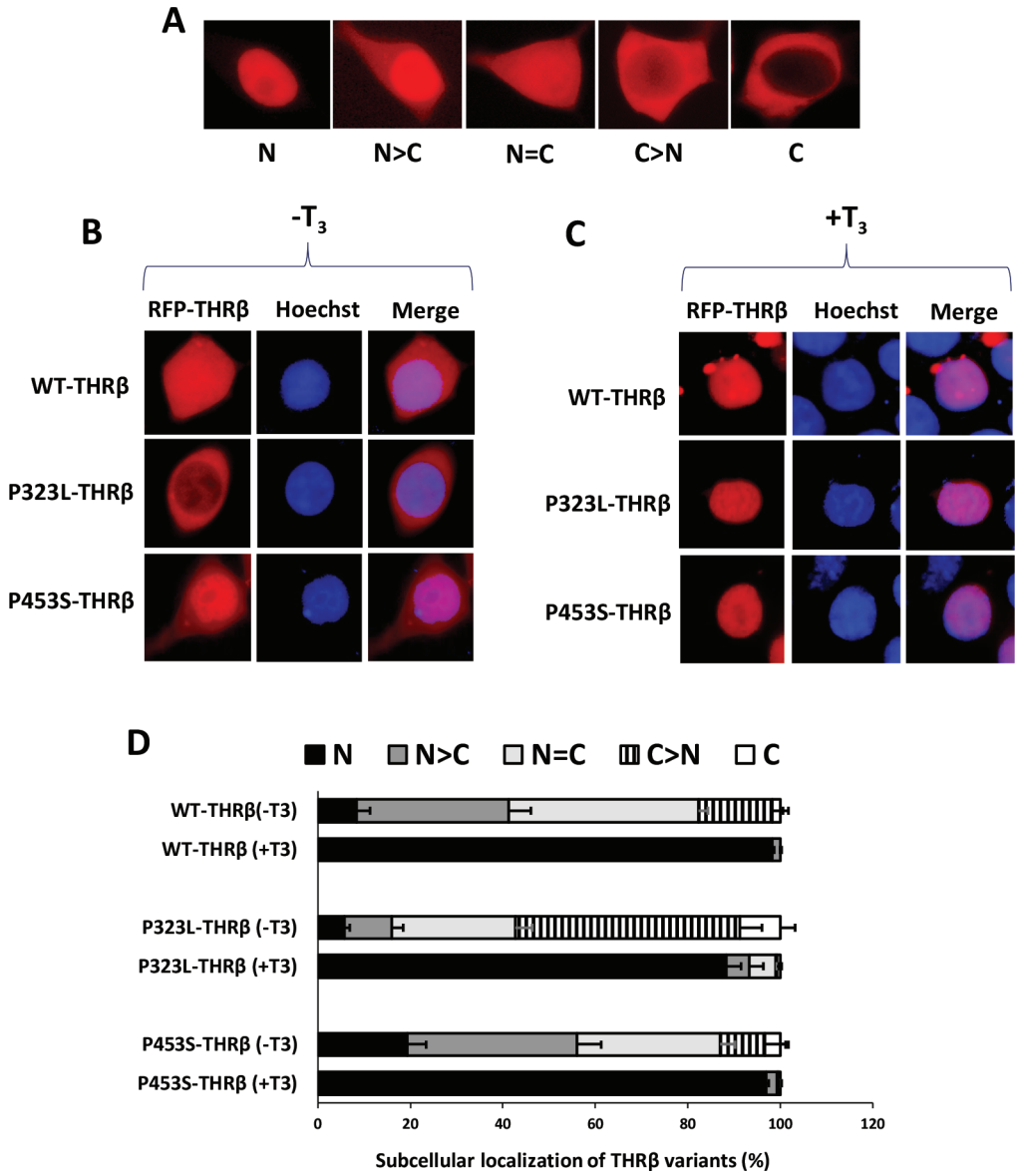


Figure 1. THRβ variants exhibit deviant subcellular localization patterns in living cells.
HEK-293T cells were transiently transfected with RFP-tagged wild-type THRβ and its THRβ variants. Images were recorded using a fluorescence microscope equipped with water immersion objectives. Hoechst was used for visualization of the corresponding nuclei. (A) The RFP fluorescence visualizes the distribution patterns (N, N>C, N=C, C>N, and C) of the receptor. Images (B) and (C) show the subcellular localization of WT-THRβ, P323L, and P453S variants in the absence and presence of T_3 hormone. (D) The bar graph shows the quantitation of subcellular localization of THRβ variants with and without T_3. In each case, localization of THRβ was recorded in at least 100 transfected cells. The graph also indicates the mean ± SE of three independent experiments.

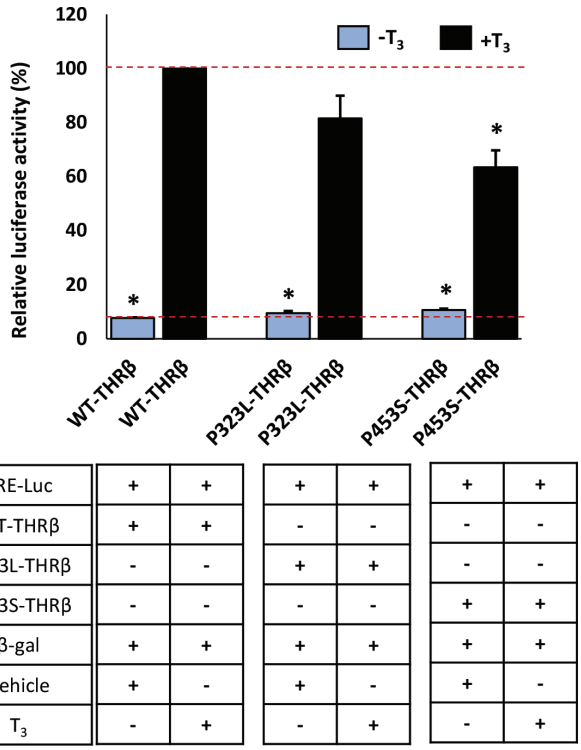
wild-type THRβ was uniformly distributed between the nucleus and cytoplasm. However, in a hormone-free state, both the disease-associated THRβ-LBD variants exhibited deviated subcellular localization of receptors (Figure 1 B, D). The THRβ variant, P323L, was observed to be cytoplasmic-shifted when compared with its wild-type counterpart. On the contrary, the other variant, P453S, appeared translocated to the nuclear compartment as predominantly nuclear-shifted. Thyroid hormone (T_3), which acts as a cognate ligand for THRβ, exclusively shifted the wild-type RFP-tagged THRβ to the nucleus. Similarly, both the variants translocated to the nucleus in the presence of the T_3 hormone (Figure 1 C, D). The observations reveal that unliganded variants of the receptor localize differently as compared to the wild-type counterpart. However, they respond to the cognate ligand similar to the wild-type receptor. Whether these variants differ in the transcription function or not need to be examined?

3.3 THRβ Variants Exhibit Altered Transcriptional Function

We performed a promoter-reporter-based luciferase assay to analyze the transcriptional response of RTH- and ASD-associated THRβ variants. The cells were transiently transfected with each THRβ construct along with TRE-Luc, followed by solvent or T_3(100 nM) treatment. After 24 h of hormone treatment, a luciferase reporter assay was conducted. The relative luciferase activity of the wild-type and its two variants was calculated and plotted. The activity of wild-type THRβ upon T_3 treatment was considered 100 %. Compared to the wild-type receptor, the transcriptional response of P323L-THRβ and P453S-THRβ was substantially reduced (Figure 2). The wild-type and the variants show differences in the subcellular localization patterns and their transcription function. These distinct behaviours of the variants may be reflective of the primary molecular determinants in disease pathogenesis.

3.4 THRβ Variants Retain Interaction with Heterodimeric Partner RXR

Class II NRs (THR, VDR, PPAR, RAR, etc.) primarily heterodimerize with partner retinoid X receptor (RXR)[24]. In some instances, with nuclear receptors, it has been observed that some disease-inflicting variants fail to interact with their heterodimeric partner, RXR[25,26]. Therefore, it is reasonable to examine if these polymorphic variants compromise their interaction with RXR. For this purpose, RFP-tagged wild-type or polymorphic variants of THRβ were co-expressed with GFP-tagged RXR. Live-cell imaging was performed in the absence and presence of the T_3 hormone. As reported earlier, GFP-RXR is exclusively localized in the nucleus[18]. When RFP-THRβ was expressed alone, it showed an N=C subcellular localization pattern; however, it shifted to the nucleus when co-expressed with RXR. Interestingly, the THRβ variants that exhibited deviant localization when expressed alone showed an RXR-dependent nuclear translocation, similar to the wild-type THRβ (Figure 3).



TRE-Luc	+	+	+	+	+	+
WT-THRβ	+	+	-	-	-	-
P323L-THRβ	-	-	+	+	-	-
P453S-THRβ	-	-	-	-	+	+
β-gal	+	+	+	+	+	+
Vehicle	+	-	+	-	+	-
T_3	-	+	-	+	-	+

Figure 2. THRβ variants exhibit reduced transcriptional activity. HEK-293T cells were transfected with different constructs of RFP-THRβ with TRE-Luc. After 4 h of transfection, the cells were treated with solvent or 100 nM T_3 hormone, according to the scheme depicted in the figure. The reporter assay was conducted after the 24-hour treatment period. The relative luciferase activity was determined by normalizing the luciferase values to β-gal values and comparing them to the RFP-THRβ activity induced by the T_3 hormone and considered as 100%. The average values from three independent replicates were plotted with ± SE. Asterisks (*) indicate the luciferase values that are significantly different from T_3-mediated RFP-THRβ expressing cells ($p \leq 0.05$).

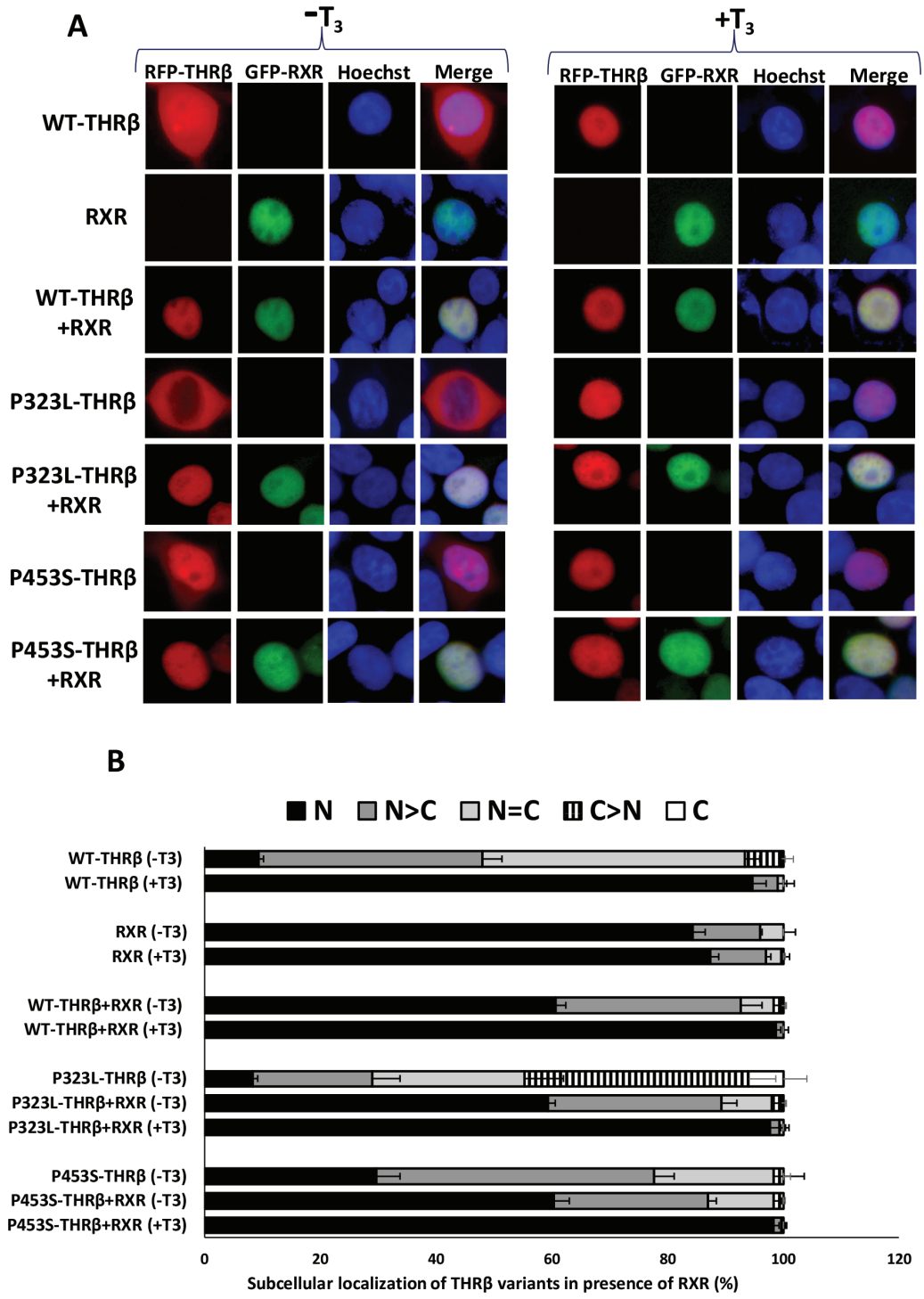


Figure 3. RXR promotes the nuclear localization of THRβ variants through heterodimeric interactions. RFP-THRβ and GFP-RXR constructs were transiently transfected alone or in combination in HEK-293T cells as per the scheme shown in the figure. Live-cell imaging was performed in the presence and absence of the T_3 hormone. Hoechst was added to stain the nucleus at least two h prior to imaging in living cells. Cell image **(A)** shows the subcellular localization pattern of THRβ variants in the presence of heterodimeric partner RXR and with or without T_3 hormone. Graph **(B)** represents the quantification of the subcellular localization of different receptor variants in the presence of RXR. Data is shown only for unliganded variants when expressed alone. The graph indicates the mean ± SE of three independent experiments.

Overall, the findings suggest that the dampened transcriptional response of these disease-inflicting variants of THRβ might be a causative factor for the disease biogenesis, along with minor altercations in subcellular localization patterns and heterodimeric partner interactions.

4. Discussion

Thyroid hormone regulates various molecular and cellular processes via nuclear receptor THR. THRβ maintains optimal circulating TH levels by negative HPT axis feedback loop and plays a crucial physiological role in metabolism, growth, and development. Polymorphisms in THRβ can cause clinical manifestations of certain diseases such as RTHβ (characterized by high TH levels, either normal or raised TSH levels), goiter, cancer as well as neurological disorders (ASD)[27].

The polymorphic variants, P323L and P453S, are in a hot spot region of LBD of THRβ found in Indian patients. In the present study, we explored the functioning of these two disease-inflicting THRβ variants by investigating their subcellular localization, response to the T_3 hormone, transcriptional response, and interaction with its heterodimeric partner, RXR. Through our investigation, we have highlighted the impairment of THRβ variants in both their subcellular localization pattern and transcriptional response. This valuable insight furthers our understanding of the cellular mechanism underlying dysfunction in diseased states. A model that summarizes the parameters to investigate the impaired cellular behaviour of RTH and ASD-associated THRβ variants is shown in Figure 4.

The deviations observed in the subcellular localization pattern and transcriptional response can be attributed

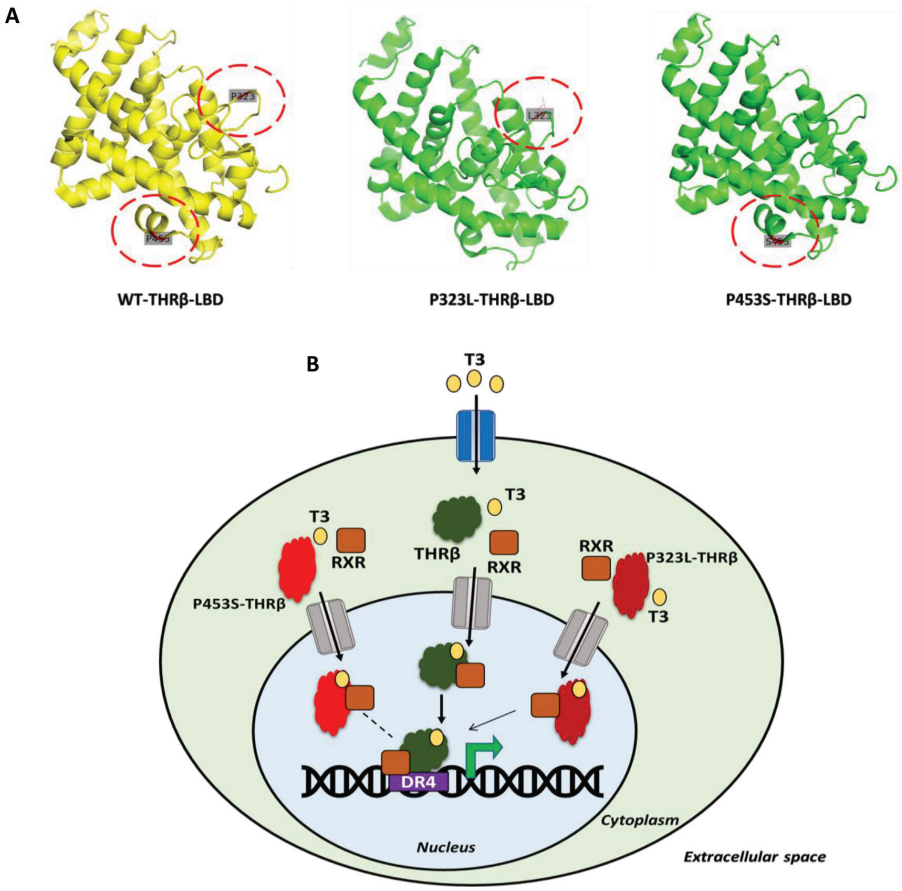


Figure 4. Graphical summary of THRβ receptor function attributed to disease-inflicting polymorphic variants. **(A)** Three-dimensional (*3D*) structures of WT-THRβ-LBD and its polymorphic variants are shown by ribbon structures. **(B)** The schematic image highlights the cellular behaviour and dynamic molecular events (hormone-receptor interaction, nuclear translocation, and gene transcription) of WT-THRβ and disease-inflicting polymorphic variants of THRβ. Note: The bold arrow indicates strong transactivation, the thin arrow suggests moderate transactivation, and the dotted arrow shows reduced transactivation.

to the polymorphisms identified in THRβ, rendering them defective or malfunctioning variants. These findings elucidate the underlying reasons behind the ineffectiveness of TH treatment in these individuals. Overall, we attempted to examine whether the structural and functional behaviour of introduced polymorphism affects normal THRβ functioning. *In silico* and several cellular parameters were considered to examine the aberrant behaviour of these polymorphic variants. The observations indicate that genetic variations, whether polymorphisms or mutations, in the *THRB* gene, express mutant variants that may negatively impact the receptor's native conformation, compromising their normal transcription function[28]. Such molecular insights will enable us to elucidate how THRβ polymorphisms contribute to the pathophysiology of THRβ-mediated diseases. It is anticipated that deciphering the molecular determinants in receptor malfunctioning may eventually lead to improved diagnosis and treatment options.

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