Functional characterization of L440Xfs mutation in the thyroid hormone receptor beta (TRβ) in an individual with RTH syndrome

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Abstract

Resistance to thyroid hormone is an autosomal dominant disorder that affects 1 in every 40,000 births. It is characterized by reduced soft tissue responsiveness to thyroid hormone, with increased levels of T4 and T3 and non-suppressed thyroid hormone (TSH), due to mutations present in the thyroid hormone receptor β gene (TRβ), particularly in its T3 binding domain. The clinical phenotype varies both between different families and between affected family members. Individuals with RTH can have variable resistance in different tissues, as a consequence of mixed features of hypo-and hyperthyroidism. In this study, we performed molecular and functional characterization of the L440Xfs mutation found in a male patient, who was diagnosed with RTH at 15 months of age. The patient harbors a new mutation in exon 10 of the TRβ gene, which consists of a deletion of a cytosine at nucleotide 1609 in the position 440, leading to a stop codon. The mutation was found in neither his parents nor his two healthy sisters, indicating a *de novo* mutational event. Transfection studies showed that the mutant TRβ was unable to carry out the transcription of luciferase gene in the presence of T3. Therefore, it is likely that the impaired receptor generates the severe RTH phenotype in our propositus.

Key words: thyroid receptor, thyroid hormone, resistance to thyroid hormone, HPT axis.

Introduction

Resistance to thyroid hormone (RTH) is a syndrome of autosomal dominant inheritance characterized by reduced responsiveness of target tissues to the active form of thyroid hormone; triiodothyronine (T3) [1]. It was identified by Dr. Samuel Refetoff in 1967 [2]. Although the precise incidence of RTH is unknown as it is usually not detected by routine neonatal screening, it has been reported that this syndrome affects 1 in every 40,000 live births and occurs with equal frequency in both sexes [3]. RTH is widely distributed since several cases have been reported in Caucasians, Africans, and Asians, nonetheless the prevalence varies among different ethnic groups.

The clinical phenotype of RTH is variable between different families and between affected family members. Actually, an individual with RTH may have variable resistance in different tissues as a consequence of mixed features of hypo-and hyperthyroidism [4]. In spite of this, the most common symptoms found in patients with RTH are: goiter, hyperactivity, tachycardia, attention deficit disorder, hearing impairment and delayed bone growth [5].

The RTH syndrome causes increased levels of thyroid hormones; triiodothyronine (T3) and thyroxine (T4), and inappropriately normal or slightly elevated thyroid-stimulating hormone (TSH) [6] also called thyrotropin, reflecting resistance within the hypothalamic-pituitary-thyroid axis (HPT). Usually, the hypothalamus senses low circulating levels of T3 and T4 hormones and responds by producing thyrotropin-releasing hormone (TRH). The TRH then stimulates the pituitary gland to produce thyroid-stimulating hormone (TSH). The TSH, in turn, promotes the production of thyroid hormones through stimulation of thyroid gland, until levels in the blood return to normal. In addition, thyroid hormones regulate the HPT axis exerting a negative feedback control which decreases the amount of TRH and TSH released from hypothalamus and pituitary gland, respectively [7] (Figure 1). However,

patients with RTH have an impairment of the HPT axis generated by non suppression of TSH and the excessive production of T3 and T4, as a result of the decreased sensitivity of thyroid hormone receptor β gene (TRβ) to T3. It is important to note that thyroid receptor silences gene expression in the absence of T3, thus the interaction between TRβ protein and the T3 hormone is essential in the regulation of target genes because this binding causes the detachment of corepressors, increasing the affinity for coactivators, allowing transcription to occur [8]

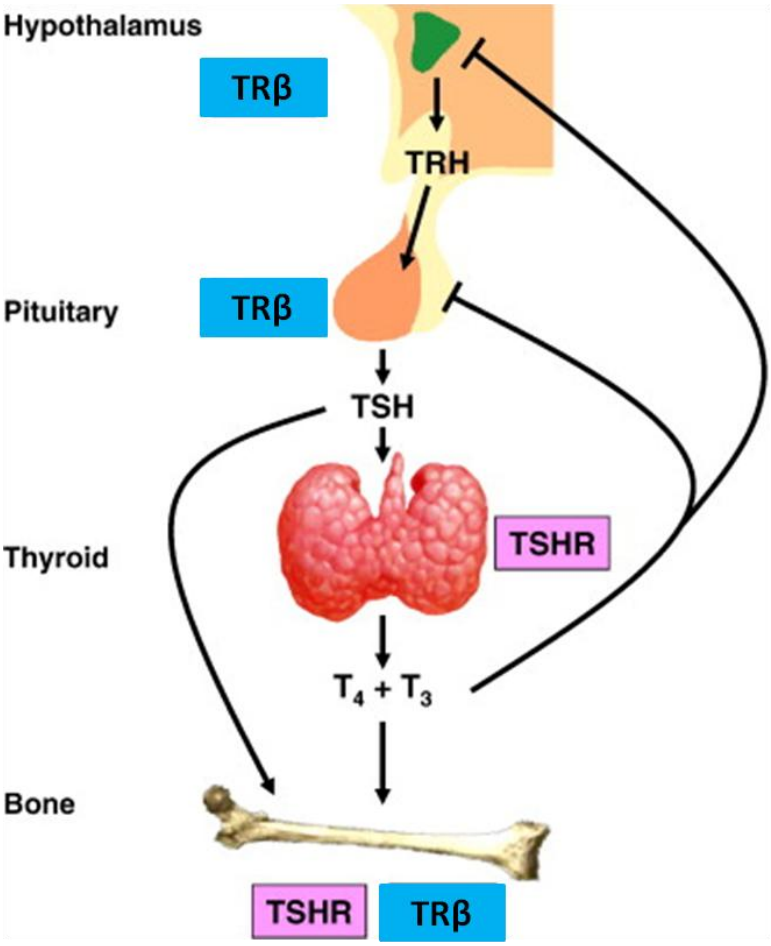

Figure 1. Hypothalamus, Pituitary, Thyroid axis [28].

The TRβ is composed of two essential domains; the DNA binding domain (DBD) and the T3 or ligand binding domain (LBD) [9]. On the one hand, the DBD allows the

binding of TRβ to specific sequences of DNA termed thyroid response elements (TREs), which are located in promoters of target genes [10], this binding lead to the regulation of transcription of genes involved in brain development, hearing, bone growth, morphogenesis, metabolism and heart rate [11]. On the other hand, the LBD is located in the carboxyl terminal which is encoded by exons 7 to 10, where the majority of mutations have been found [12], [13], [14] (Figure 2). Furthermore, this is the domain where T3 binds, however studies have demonstrated that mutant TR proteins have reduced ability to bind T3, affecting the transcriptional activity [6].

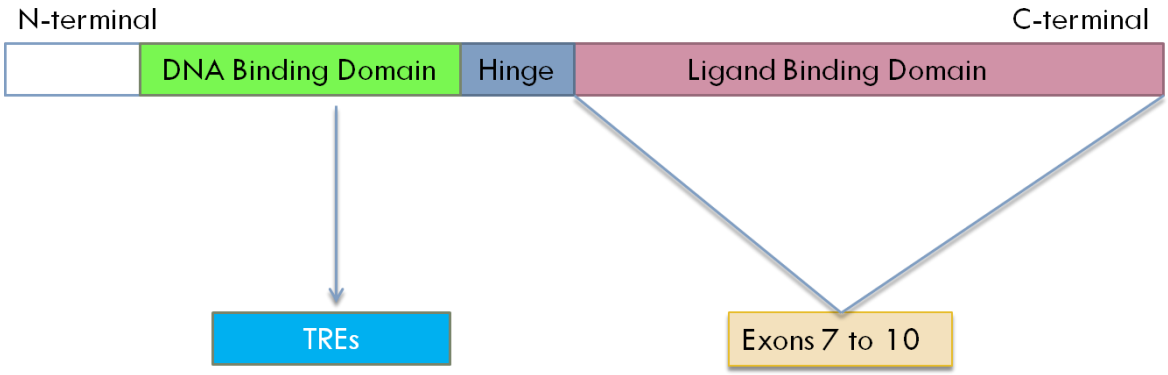

Figure 2. Thyroid hormone receptor β gene.

According to previous reports, 80% -90% of individuals with RTH have mutations in the carboxyl terminal of TRβ. In fact, in 2002 [15], there were 300 families evaluated, in which 122 mutations were found; 285 families presented single nucleotides substitutions resulting in single amino acid replacements in 281 instances and stop codons in the remaining, producing truncated proteins. In 2 families dinucleotide substitutions produced a single amino acid change and a premature stop, respectively. Besides, 6 families showed trinucleotide deletion producing the loss of single amino acid and another family presented an addition of a single amino acid due to an insertion of a trinucleotide. Finally

there was found a single nucleotide deletion in 1 family and an insertion in 4 others, resulting in a frameshift.

Moreover, in 2010, 1000 RTH cases had already been published. In this year, Weiss et al., studied 183 of 372 families with RTH. Of these families, 85% had mutations in the TRB gene and 28% of mutations were *de novo* [16]. Nevertheless, there are some RTH cases reporting no mutations in the TRB gene, but mutations in coactivators and corepressors that are part of the transcriptional machinery of TRβ [16].

Currently, in Colombia there are no reports associated to RTH; one of the main reasons is that the diagnosis is erroneous at the time of neonatal screening, confounding RTH with other thyroid diseases such as hyperthyroxinemia or thyrotoxicosis. This is mostly due to the lack of information and knowledge of the syndrome by the medical community.

Our research group performed a molecular characterization of 8 families searching for mutations in exons 7 to 10 of the TRB gene. We found a mutation in exon 10 of the propositus of family 8; therefore in this investigation our main objective was to assess whether there was a functional alteration due to that mutation (L440Xfs) in TRβ gene that can generate RTH. Thus, we compare the transcriptional activity between wild type TRβ and mutant TRβ through luciferase activity, in the presence and the absence of T3.

Materials and Methods

- Case Report

The propositus is a male from Cali, Valle del Cauca, born at 38 weeks gestation to healthy nonconsanguinous parents. He was referred by Dr. Liliana Mejía, from Fundación Valle. The patient´s birth weight was 2.4 kg and his height was 46 cm, values within the

normal range. At 8 months of age, psychomotor delay was evidenced and the fontanelle was closed at 9 months of age (craniosynostosis). The child crawled at 15 months and walked at 22 months, supporting the diagnosis of psychomotor retardation. Besides, patient was noted to have hearing loss, visual impairment, hypotonia and seizures, although tachycardia was not present. Total T3 and T4 serum levels were extremely high, and his TSH levels were normal (Table 1).

Table 1. Results of thyroid function tests for the propositus at the time of study.

	Patient	**Normal Range**
TSH	2.7mU/L	0.4-3.6 mU/L
Total T3	8000 ng/dl	75-180 g/dl *
Total T4	>30µg/dl	4-12 ug/dl*

***** [17]

- Genetic Analysis

Informed consent and assent was fully explained and signed by the child´s parents in order to perform the genetic analysis. Genomic DNA was extracted from peripheral white blood cells of the propositus and members of his family, using the Corpogen DNA 2000 kit (Corpogen, Colombia). Then, exons 7 to 10 of the TRβ gene of the propositus were amplified by PCR. Primers previously reported in the literature [18] were used at an annealing temperature of 60°C (Table 2). The PCR product was confirmed by direct sequencing (Macrogen, Korea). The sequence was compared to the consensus sequence obtained from NCBI (National Center of Biotechnology Information) using Bioedit Sequence Alignment Editor.

Table 2. Primers used for amplification of exons 7-10 of gene TRβ

Exon	Forward Primer	Reverse Primer
7	5´CATCAGTGGTCCCACTCCTG 3´	5´CACCAGTATCCCAAGGTGATG 3´
8	5´GTTCAGAAGAGATTTTCTGCC 3´	5´TCGTTTTGTACTGACGTTGC 3´
9	5´GGAAAACCATGGGCTCAAAG 3´	5´AGCGCTAGACAAGCAAAAGC 3´
10	5´TAAAGGCCTGGAATTGGACA 3´	5´CTACTTCCCTTTTCCCTCCCA 3´

- Plasmids

The expression vector pcDNAI/Amp-TRβ and the reporter vector Palx3-Luc were kindly donated by Dr. Refetoff from University of Chicago.

- Expression vector (pcDNAI/Amp-TRβ): wild type TRβ cDNA is inserted at BamHI and EcoRI of pcDNAI plasmid, which has ampicillin resistance.
- Reporter vector (Palx3-Luc): it expresses the firefly luciferase (Luc) gene, which has, at the upstream region three copies of a palindromic TRE (AGGTCATGACCTGAAAGCT).
- Control pRL-TK vector (Promega, USA): it expresses the Renilla luciferase (Rluc) gene. It was used as an internal control to normalize transfection variability from well-to-well in the transient transfection assays.

- Transformation

In order to obtain working material of the three plasmids, they were each transformed in E. coli DH5α with ampicillin resistance. The transformation was carried out by thermal shock, placing the reaction on ice for 30 minutes, followed by a heat pulse for 45 seconds at 42°C and a final incubation on ice for 2 minutes. Then, the transformation

reaction was incubated at 37°C in 500 µl of LB broth for 1 hour shaking at 250 rpm. Finally, 300 µl and 200 µl were placed on agar plates containing ampicillin and were incubated at 37°C for 18 hours. Plasmids purification was carried out using Minipreps columns. Expression vector clones were confirmed by enzymatic digestion with HindIII and BglII, while the clones of reporter vector were verified by direct sequencing (Macrogen, Korea).

- Mutagenesis

Two complementary oligonucleotides containing the mutation found in the exon 10 of the TRβ gene were synthesized and purified by HPLC. The primers were 30pb in length, with a melting temperature of >78°C, according to the instruction manual of QuickChange II Site- Directed Mutagenesis Kit (Agilent technologies, USA).

The synthesis of the mutant strand was performed through PCR, using 125 ng of each primer, 75 ng of wild type pcDNAI/Amp-TRβ and *Pfu* polymerase. The thermal cycling protocol is shown in table 3.

Table 3. Thermal cycling protocol for mutagenesis

Cycles	Temperature	Time
1	95°C	30´´
18	95°C	30´´
	58°C	1´
	68°C	6´13´´
1	68°C	10´

Once the PCR product was obtained, the template was digested with 1 µl of DpnI restriction enzyme (10 U/ µl), which cuts the parental methylated and hemimethylated DNA. The reaction was incubated at 37°C for 1 hour. Later, 1 µl of plasmid with the

mutation already inserted was transformed in 50 µl of supercompetent cells for nick repair. The transformation protocol was described above. Clones were confirmed by direct sequencing (Universidad de Los Andes, Bogotá, Colombia).

- Cell culture and transfection studies

COS-7 cells (simian virus 40-transformed African green monkey kidney fibroblasts) were grown in DMEM (Invitrogen, USA) containing 10% fetal bovine serum (FBS), 50 µg/ml gentamicin at 37° C in 100% humidity, and 10% CO2. Before transfection, 1 x 10^5 cells were transferred to each well of a 96-well culture plate and grown in the same medium until they reached 70%-80% confluence. On the day of transfection the growth medium was removed and cells were washed twice with Hank's buffered saline solution. Then, the transfection solution that consisted of DMEM, lipofectamine (Invitrogen), and expression and reporter plasmids was added. Furthermore, the pRL-TK vector was also transfected to normalize the results.

After 5 hours, cells were washed twice with Hank's solution and incubated for 24 hours in DMEM containing 10% thyroid hormone-stripped FBS and gentamicin. After incubation with this thyroid hormone-depleted medium, cells were grown in the presence and absence of T3 and incubated for 48 hours. The T3 dose used was 10^{-7} M. Additionally, untreated cells were cultured in parallel as negative controls. Cells transfected with pRL-TK plasmid alone were harvested as positive controls. The experiments were performed twice, with three replicates per experiment.

- Luciferase Activity

Luciferase activity was measured after 72 hours of transfection, using the Dual-Luciferase® Reporter Assay System (Promega). First, the growth medium from the cultured cells was removed and they were washed with Hank's solution. Afterwards, 20 µl

of passive lysis buffer 1X was dispensed into each culture well and the lysate was transferred to a 96-well luminometer plate. Later, 100 µl of the luciferase assay reagent II (LARII) was added to the lysate and the microplate was placed into the luminometer to perform the lectures of *Firefly luciferase* activity, subsequently 100 µl of Stop & Glo reagent was added and the second lecture was carried out, measuring the *Renilla luciferase* activity. Results were expressed as fold luciferase activity.

Results

- Identification of TRβ mutation

PCR amplification and the subsequent sequencing of exons 7 to 10 of TRβ gene, allowed the identification of a heterozygous mutation in the propositus. He harbors a new mutation in exon 10 of the TRβ gene, which consists of a deletion of a cytosine at nucleotide 1609 (codon 440) leading to a stop codon in the position 442 (Figure 3). The obtained encoded mutant receptor, which was denominated L440Xfs at protein level or 1609delC at DNA level, lacked the last 20 amino acids of the TRβ protein.

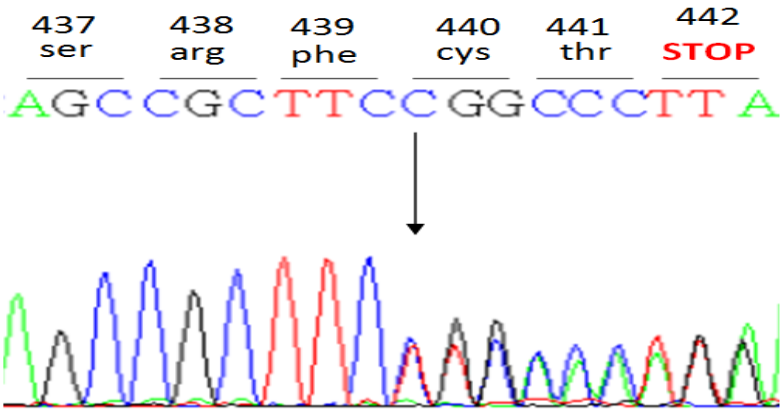

Figure 3. Direct sequencing of exon 10 of TRβ indicates that the patient is heterozygous for a de novo mutation L440Xfs

The mutation was found in neither his parents nor his two healthy sisters, indicating a *de novo* mutational event (Figure 4.)

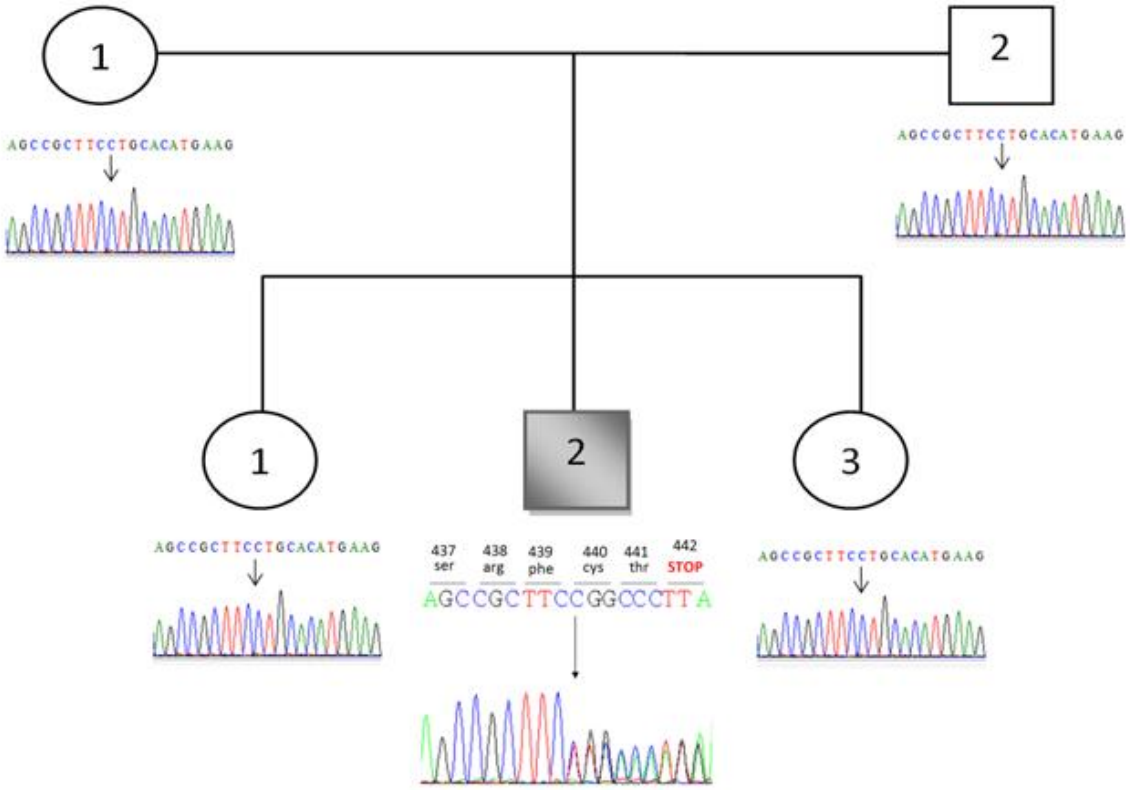

Figure 4. Family genealogy shows that the mutation is *de novo*. Neither of the parents have the mutation L440Xfs.

- Clone verification

On the one hand, the digestion of the expression plasmid (pcDNAI/Amp-TRβ) with HindIII and BglII restriction enzymes confirmed the existence of TRβ cDNA within it (Figure 5). On the other hand, the sequencing of the reporter vector corroborated the presence of TREs that are essential for the binding of the protein TRβ.

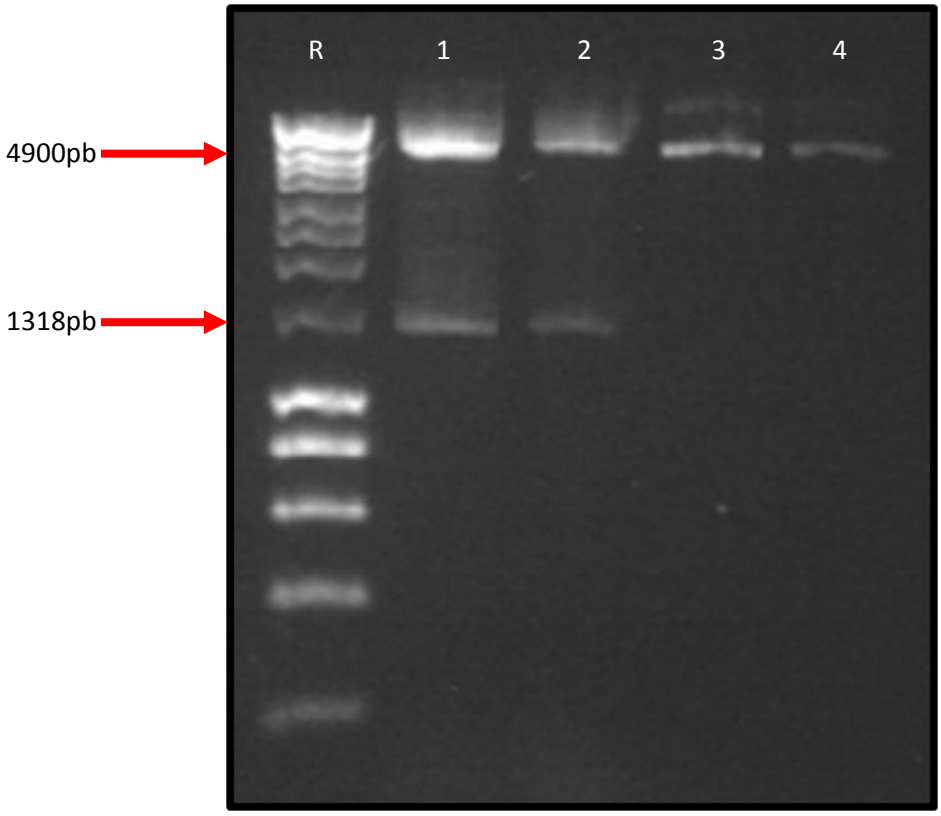

Figure 5. Digestion of pcDNAI/Amp-TRβ plasmid with HindIII and BglII. Ruler (R). Digested Plasmid (1) and (2). Undigested plasmid (3) and (4).

- Site Directed Mutagenesis of exon 10

The clones obtained after the mutagenesis were sequenced to confirm that the mutation 1609delC was inserted (Figure 6).

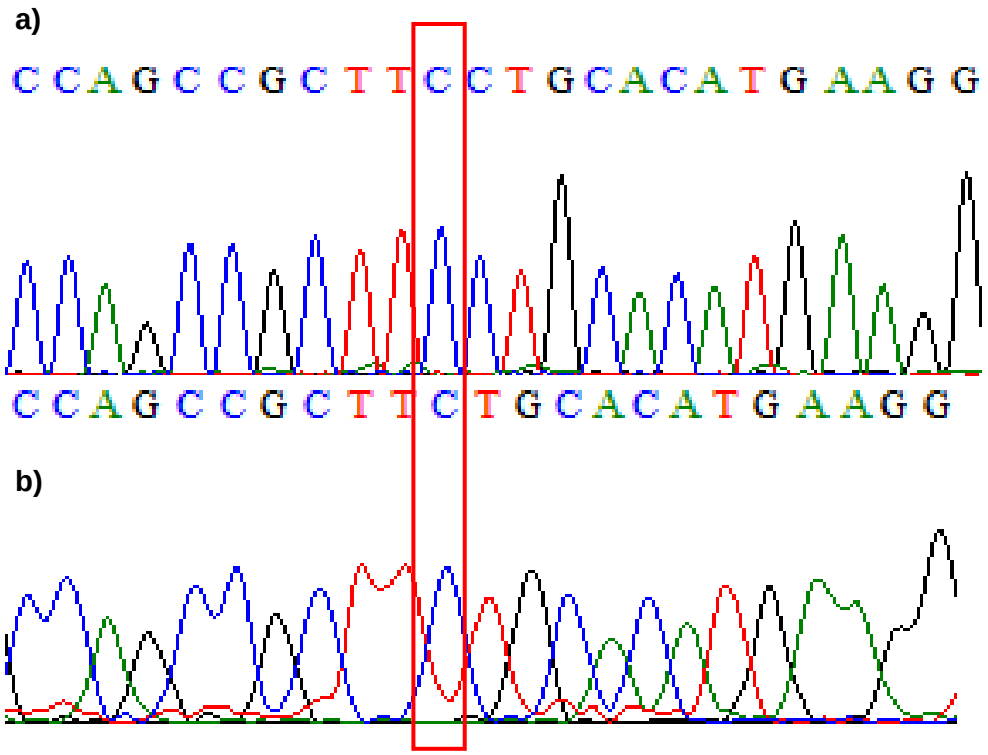

Figure 6. Direct sequencing provided evidence of cytosine deletion performed by site directed mutagenesis. a) wild type sequence b) mutated sequence.

- Functional Analysis

The experiments allowed differentiating between expressions of luciferase, regulated by TRβ protein and the binding to T3. We observed that luciferase activity was higher when the wild type TRβ was bound to T3 in comparison with the absence of it. In fact, the activity of wild type receptor bound to T3 is 3-fold above unbound receptor. On the other hand, our results provide evidence that the mutant receptor (L4404Xfs) is unable to activate the transcription of luciferase gene in the presence of T3, obtaining a very similar result in the absence of it (Figure 7). Therefore, this mutant receptor repressed the transcription of the luciferase. Thus, the mutation L440Xfs, indeed, affects the transcriptional regulation of essential genes. In addition, the ANOVA analysis showed a

significant interaction between the T3 binding and the type of TRβ receptor (mutant or wild type) obtaining a p-value of 0.002.

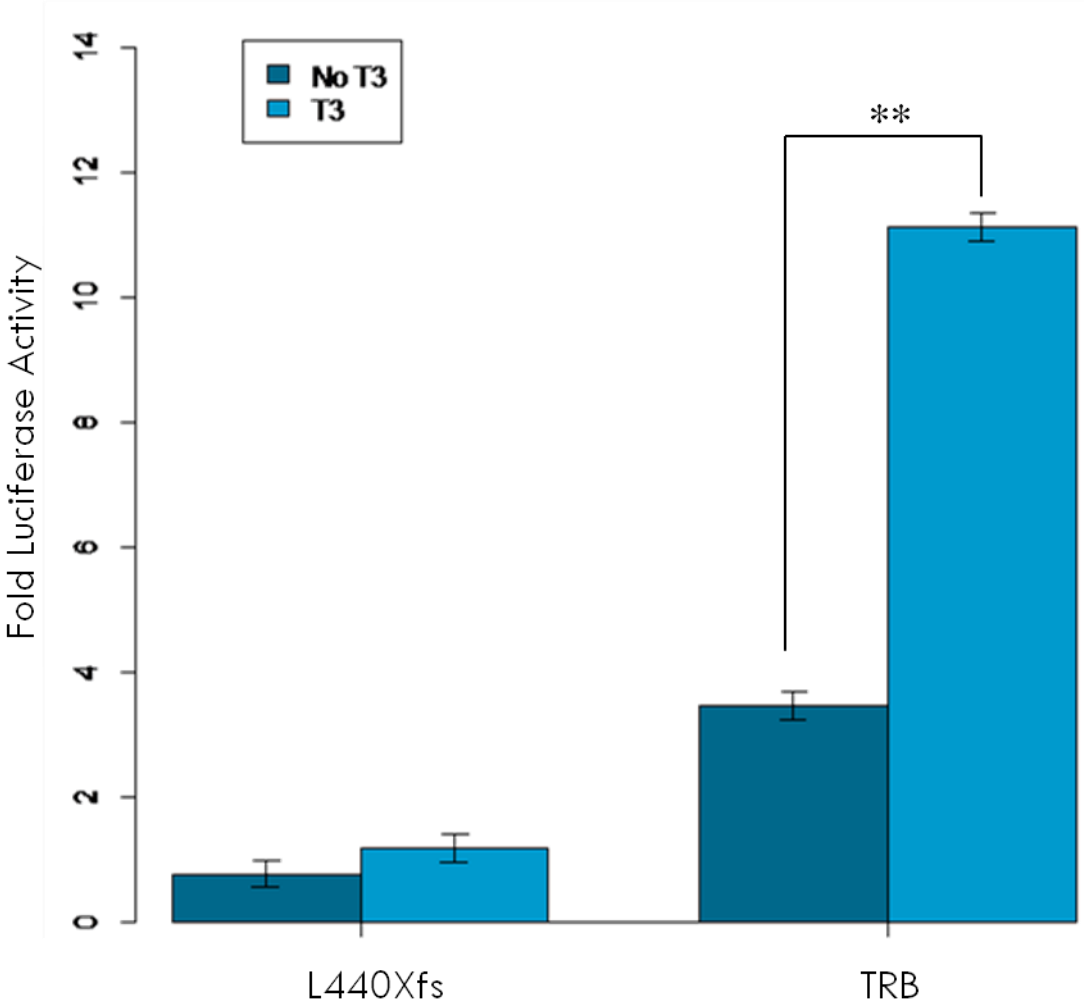

Figure 7. Transcriptional activity of the wild type and mutant TRβs in the presence and absence of T3. The L440Xfs receptor lacks transcriptional activity even in the presence of T3 ($p>0.05$). The TRβ (wild type) receptor shows 3-fold luciferase activity in presence of T3 than in absence of it ($p<0.01$).

Discussion

We identified an individual with RTH caused by a *de novo* mutation which consists in a cytosine deletion in codon 440 of the TRβ gene. The mutation, named L440Xfs, is located in the ligand binding domain (LBD) of TRβ protein and lead to a frameshift producing a stop codon and the absence of 20 amino acids. The L440Xfs mutation is comparable to the previously reported in 2001 by Phillips and collaborators [19] (Table 4). In this investigation, a deletion of a cytosine was found in nucleotide 1603 leading to a stop in the same codon that the mutation evaluated in the present study, however the change of amino acid was two codons downstream of the mutation reported by Phillips [19].

Table 4. Comparison between wild type and mutant TRβ.

Codon	437	438	439	440	441	442	443
WT (NCBI)	AGC *Ser*	CGC *arg*	TTC *phe*	CTG *leu*	CAC *his*	ATG *met*	AAG *lys*
1603delC [19]	AGC *ser*	GCT ***ala***	TCC ***ser***	TGC ***cys***	ACA ***thr***	TGA ***stop***	___
1609delC [20]	AGC *ser*	CGC *arg*	TTC *phe*	TGC ***cys***	ACA ***thr***	TGA ***stop***	___

In addition, the symptoms of our patient are very similar to the girl examined in the case mentioned above; both have a history of seizures, hearing loss, hypotonia and psychomotor retardation. An interesting characteristic is that our patient does not present

goiter as the girl does, however this difference may be due to variable clinical manifestations characteristic of RTH. Nonetheless, goiter frecuency in indviduals with RTH is 66-95% [21]. Moreover, seizures as well as hearing loss are very rare in this syndrome. In fact, hearing loss has been reported in only three members of one family homozygous for TRβ gene deletion [22]. The frecuency of this characteristic is 10-22%. Psychomotor retardation is also confirmed in RTH, this feature is related to the nervous system which also include attention deficit hyperactivity disorder, learning disability, mental retardation, etc. [21].

It is essential to note that the case reported by Phillips is recognized as one of the most severe RTH phenotype, therefore due to the similarity with the features presented by our patient, our case may be considered severe as well, regarding the extremely high levels of thyroid hormones that our propositus has.

In this study, the L440Xfs receptor was not able to regulate the expression of luciferase in the presence of T3, providing evidence of the TRβ protein alteration, in contrast with the wild type TRβ function. Therefore, the mutant receptor represses the transcription of essential genes.

There are several mutations that lead to truncated TRβ and these have been described previously in RTH patients. In 1997, Miyoshi [23] and coworkers compared the functional properties of three truncated thyroid hormone receptors with 11 (F451X), 13 (E449X) and 16 (C446X) amino acid deletions, all of these located in the LBD domain. The patients who had F451X and C446X mutations presented impaired speech development, impaired hearing and mental retardation indicating a severe phenotype. Similar to our propositus, the expression studies revealed a TRβ impairment to bind T3 and regulate transcription. Behr and collaborators [24] examined a patient with a exchange of a cytosine by adenine in exon 10, resulting in the lack of the last 28 amino acids of TRβ with no T3

binding. This patient also presented a severe phenotype with a mental and physical development extremely retarded. Besides, serum thyroid hormone levels were high, similar to our patient levels. Other studies shows that transactivation function depends on the impairment in the TRβ. In this investigation two mutant receptors with undetectable ligand affinity showed no transcriptional activity, whereas two other mutations characterized by a no total reduction in T3 affinity required higher concentrations of hormone for transcription to occur [25]. Nevertheless, we have to consider mutations located in the LBD which lead to exchange of amino acid but in spite of this they do not cause severe phenotypes because there are not deletion of several amino acids [26], [27].

In summary, the identification of the L440Xfs mutation in exon 10 of TR β and the functional characterization, enhances our understanding of the biological processes that are regulated by thyroid hormones. Due to the variability of phenotypes in individuals with RTH syndrome it is important to achieve an accurate diagnosis in patients with abnormal thyroid tests, as this will determine the treatment that doctors should follow at critical stages of neurodevelopment. We must continue in the effort to characterize this population of patients in order to refine the diagnosis and optimize the treatment of this pathology.

Finally , in Colombia, so far, there are not reported mutations associated with RTH, thus the mutation found in this study it is a starting point to carry on researching this syndrome in our country. It is noteworthy that it is not enough to discover these mutations but also is essential to carry out the functional characterization of mutant TRβ proteins, in order to better understand the pathology of RTH in these individuals which allow to offer appropriate genetic counseling, given the importance of thyroid hormones and the TRβ in the development and maintenance of body homeostasis.

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