Patient report

Taise Lima Oliveira Cerqueira, Aurore Carré, Lucie Chevrier, Gabor Szinnai, Elodie Tron, Juliane Léger, Sylvie Cabrol, Chrystelle Queinnec, Nicolas De Roux, Mireille Castanet, Michel Polak and Helton Estrela Ramos*

Functional characterization of the novel sequence variant p.S304R in the hinge region of TSHR in a congenital hypothyroidism patients and analogy with other formerly known mutations of this gene portion

Abstract

Context: Thyroid dysgenesis may be associated with loss-of-function mutations in the thyrotropin receptor (*TSHR)* gene.

Objectives: The aim of this study was to characterize a novel *TSHR* gene variant found in one patient harboring congenital hypothyroidism (CH) from a cohort of patients with various types of thyroid defects.

Materials and methods: This cross-sectional cohort study involved 118 patients with CH and their family members, including 45 with familial and 73 with sporadic diseases. The thyroid gland was normal in 23 patients, 25 patients had hypoplasia, 25 hemithyroid agenesis, 21 had athyreosis, and 21 had ectopy. Genomic DNA was extracted, and 10 exons of the *TSHR* gene were amplified and sequenced. Mutations in other candidate genes were investigated. Ortholog alignment was performed, and *TSHR* functional assays were evaluated.

Results: We identified one previously unknown missense variation in the hinge region (HinR) of the *TSHR* gene (p.S304R) in one patient with thyroid hypoplasia. This variant is conserved in our ortholog alignment. However, the p.S304R *TSHR* variant presented a normal glycosylation pattern and signal transduction activity in functional analysis.

***Corresponding author: Helton Estrela Ramos,** Federal University of Bahia, Health and Science Institute, Department of Biorregulation, Avenida Reitor Miguel Calmon, S/N, Sala 301, Vale do Canela, 40110-102 Salvador, Bahia, Brazil, Phone: +55 71 3283 8890, Fax: +55 71 3283 8927, E-mail: ramoshelton@gmail.com, http://orcid.org/0000-0002-2900-2099; Curso de Pós-Graduação em Biotecnologia em Saúde e Medicina Investigativa, Centro de Pesquisa Gonçalo Moniz-FIOCRUZ/BA, Salvador, Bahia, Brazil; INSERM U845, Université Paris Descartes, Sorbonne Paris Cité, Paris, France; Pediatric Endocrine Unit, Centre des Maladies Endocriniennes Rares de la Croissance, Hôpital Necker Enfants Malades, AP-HP, Paris, France; and Departamento de Biorregulação, Instituto de Ciências da Saúde, Universidade Federal da Bahia, Salvador, Bahia, Brazil

Taise Lima Oliveira Cerqueira : Curso de Pós-Graduação em Biotecnologia em Saúde e Medicina Investigativa, Centro de Pesquisa Gonçalo Moniz-FIOCRUZ/BA, Salvador, Bahia, Brazil; and Departamento de Biorregulação, Instituto de Ciências da Saúde, Universidade Federal da Bahia, Salvador, Bahia, Brazil

Aurore Carré and Elodie Tron: INSERM U845, Université Paris Descartes, Sorbonne Paris Cité, Paris, France

Lucie Chevrier and Nicolas De Roux: Pediatric Endocrine Unit, Hôpital Armand Trousseau, AP-HP, Paris, France

Gabor Szinnai: Pediatric Endocrinology, University Children's Hospital Basel, University Basel, Switzerland

Juliane Léger: Pediatric Endocrine Unit, Centre des Maladies Endocriniennes Rares de la Croissance, Hôpital Robert Debré, AP-HP, Paris, France

Sylvie Cabrol: Pediatrics department, CHU, Bordeaux, France

Chrystelle Queinnec: Pediatrics department, CH de Cornouailles-hopital Laennec, Quimper, France

Mireille Castanet: INSERM U845, Université Paris Descartes, Sorbonne Paris Cité, Paris, France; Pediatrics department, CH Charles Nicolle, University Hospital of Rouen, Rouen, France; and Pediatric Endocrine Unit, Centre des Maladies Endocriniennes Rares de la Croissance, Hôpital Necker Enfants Malades, AP-HP, Paris, France

Michel Polak : INSERM U845, Université Paris Descartes, Sorbonne Paris Cité, Paris, France; and Pediatric Endocrine Unit, Centre des Maladies Endocriniennes Rares de la Croissance, Hôpital Necker Enfants Malades, AP-HP, Paris, France

Conclusion: We report the ocurrence of a novel nonsynonymous substitution in the HinR of the large N-terminal extracellular domain of the *TSHR* gene in a patient with thyroid hypoplasia. In contrast with four others in whom TSHR mutations of the hinge portion were previously identified, the p.S304R *TSHR* variation neither affected TSH binding nor cAMP pathway activation. This *TSHR* gene variant was documented in a CH patient, but the current data do not support its role in the clinical phenotype.

Keywords: congenital hypothyroidism; hinge portion; thyroid dysgenesis; TSH receptor; variants.

DOI 10.1515/jpem-2014-0194
Received May 13, 2014; accepted July 14, 2014; previously published online August 19, 2014

Introduction

Thyrotropin (TSH) exerts its effects on the thyroid cell by binding to specific receptors on the cell surface (1). The thyrotropin receptor (TSHR) belongs to the superfamily of seven-transmembrane receptors that trigger signal-transduction pathways through their synergy with guanine-nucleotide-binding regulatory proteins (G proteins) (1–4). As such, they contain a canonical serpentine region containing seven transmembrane helices typical of rhodopsin-like glycoprotein hormone receptor (GPCRs), and a large (350–400 residues) amino-terminal ectodomain containing leucine-rich repeats, which is responsible for the high affinity and selective binding of the corresponding hormones (1). The amino-terminal segment is responsible for high affinity binding of the hormones and recognition specificity (5). The hinge region (HinR) is the least conserved GPCR portion and the most variable in size, between the extracellular leucine-rich repeat motif and the transmembrane helices (6–10). The importance of this region for hormone binding, signal transduction, and receptor activation has been indicated in several studies; however, the hormone TSHR interaction mechanisms have yet to be completely understood (8–10). The intramolecular transduction mechanism of the signal, by which binding of TSH to the extracellular region of the receptor is transduced via the membrane-spanning regions to the cytoplasmic compartment of the receptor that interact with the G proteins, is not well known. A current model for the activation of TSHR would examine a zone of discrete states characterized by different configurations (2, 3).

In the frame of our discussion, it is worth noting that amino acid substitutions in the ectodomain of GPCRs might affect their function by modifying the electrostatic surface map of the variant receptor, thereby leading to a loss of specificity, or affinity through a shift on sialylated and sulfated carbohydrate structure (1, 11).

Germline TSHR loss-of-function mutations in the *TSHR* gene have been involved as the molecular answer for TSH resistance and congenital hypothyroidism (CH) (OMIM no. 275200). Subjects with a heterozygous loss of function mutation appear to have a dominant transmission of partial TSH resistance, which is attributed to intracellular entrapment, reduced maturation, and oligomerization between inactive mutants and the WT receptor (12–16). Indeed, in the past years, many germline TSHR variants, culminating in amino acid substitutions have been discovered (12). Two of these are located in the extracellular domain of the receptor (p.D36H and p.P52T), and one is located in the intracellular domain (p.D727E) (17–19). The *TSHR* variant p.D727E allele associated with lower levels of plasma TSH without effect on FT_4 levels, point toward a higher sensitivity of the variant compared with the WT *TSHR*. This is because less TSH is needed to achieve normal FT_4 levels, as reported in two studies (20, 21). However, others have not been able to reproduce such data (22, 23). Despite the fact that the p.P52T and p.D36H polymorphisms have not been correlated with modifications in serum TSH levels in healthy individuals, conflicting data are also available regarding the response of the extracellular domain p.P52T variant to TSH stimulation (24, 25).

In the current study, we report a novel germline variant, p.S304R, located in the hinge region (HinR) of the extracellular domain of the TSHR, which is present in heterozygocity in a patient with congenital hypothyroidism. We examined the functional properties of this variant by transient expression in HEK293 cells, with particular interest in the effect on binding ability, cAMP production, and glycosilation profile. The knowledge about the contribution of systematically conserved residues within the TSHR HinR to hormone binding is very important in the further clarification of their precise functional importance in hormone-receptor interactions.

Materials and methods

Subjects

We enrolled 118 patients with CH, including 45 with familial and 73 with sporadic diseases recruited from the French Neonatal Screening Program, along with their family members (Table 1). The thyroid gland was normal in size and position in 23 of these patients; 92 patients had hypoplasia (n=25), hemithyroid agenesis (n=25),

Table 1 Patient characteristics.

Variable	
Number, male, female	118 (47, 71)
Age, year, median, range	11.0 (1.1–23.2)
Family history of CH, n	45
Sporadic cases of CH, n	73
Blood-spot TSH at screening, mU/L, median, range	72.9 (16.9 to >200)
Thyroid morphology, n	
Ectopy	21
Athyreosis	21
Hypoplasia	25
Hemithyroid	25
Normal	23
Not evaluated	03
Kidney malformation	07

CH, congenital hypothyroidism.

athyreosis (n=21), or ectopy (n=21); and thyroid gland morphology was unknown in three patients. Informed consent was obtained from all patients and their families, and blood samples were collected. The study was approved by the local Ethical Committee.

TSHR sequencing

Genomic DNA was extracted from whole blood, and 10 exons of the *TSHR* gene were amplified in 15 separate PCRs (26). PCR products were purified using the Qiaquick PCR purification kit (QIAGEN, Hilden, Germany) and sequenced using the ABI PRISM Dye Terminator cycle sequencing Ready Reaction kit (PE Applied Biosystems, Foster City, CA, USA) according to the manufacturers' instructions. Sequences were analyzed using Sequence Navigator Software (PE Applied Biosystems Division, Perkin Elmer, Foster City, CA, USA). Bidirectional sequencing was performed using an automated cycle sequencer (ABI) Prism 3100 Genetic Analyzer (Applied Biosystems, Vernon Hills, IL, USA). Sequence alterations were examined in the context of the open reading frame to determine whether the alteration changed the corresponding amino acid. A total of 100 normal individuals were screened for the identified sequence alterations. Mutations in other candidate genes (*PAX8, NKX2.1, NKX2.5*) were excluded.

Annotation of the human TSHR protein

Genbank accession no. NP_16473 (www.ncbi.nlm.nih.gov) was used for the human TSHR protein reference. Exon positions were calculated from the annotation at the University of California, Santa Cruz, Genome Browser (http://genome.ucsc.edu).

Prediction of TSHR orthologs and alignment with human TSHR sequence

The corresponding proteins for each of the 11 non-human species (*Pan troglodydes, Macaca mulatta, Rattus norvegicus, Mus musculus, Bos taurus, Dasypus novemcinctus, Monodelphis domestica, Gallus gallus, Xenopus tropicalis, Takifugu rubripes and Orysias latipes*) used in this study were obtained as described here. First, the genomic sequence was obtained from all species; second, protein predictions were obtained for each DNA sequence using GENSCAN software (http://bioweb.pasteur.fr/seqanal/interfaces/genscan-simple.html) (27) and default parameters; third, results from GENSCAN were compared with the NP_16473 reference sequence using BLAST (www.ncbi.nlm.nih.gov) (28) to identify TSHR orthologs; and fourth, TSHR protein orthologs were aligned to the human reference using CLUSTALW (http://clustalw.genome.ad.jp) (29). Accession numbers corresponding to all sequences used in this study are provided under solicitation.

TSHR-S304R functional studies

Western-blot analysis and ^{125}I-TSH binding experiments were performed as previously described (30). Accumulation of cAMP was measured using the cAMP dynamic 2 kit (Cisbio Bioassays, Bagnols-sur-Cèze, France) according to the manufacturer's instructions. Transient HEK293-cell transfection with plasmid-encoded *TSHR*-WT or *TSHR*-S304R was performed, and 48 h later the cells were washed once with phosphate buffer saline (PBS) and detached with PBS-ETDA 5 mM. The cells were then resuspended in stimulation buffer (Krebs buffer with 0.28 M sucrose) and plated in 384-well plates. The cells were stimulated for 30 min with various TSH concentrations in stimulation buffer, which was upplemented with 0.5 mM of IBMX. After stimulation, fluorophore d2-labeled cAMP and anti-cAMP antibody conjugated to cryptate were added and incubated for 1 h at room temperature, and the plate was read at 620 nm and 665 nm using the PARADIGM™ Detection Platform (Beckman Coulter, Brea, CA, USA). Data were analyzed using GraphPad Prism Software (GraphPad, San Diego, CA, USA).

Results

Genetic screening and clinical phenotype

The family pedigree has been previously described (31). Direct *TSHR* gene sequencing identified one different heterozygous *TSHR* variant (p.S304R) in three family members: the proposistus, his father, and the older brother (Figures 1 and 2A). The affected boy is the second child of healthy, nonconsanguineous parents and was born at term, following an uncomplicated pregnancy and delivery, with a weight of 3.400 kg. Neonatal TSH screening showed a TSH level of 155 μU/mL (normal <20 μU/mL) and an FT_4 level of 0.8 ng/dL (normal 0.8–1.8). He presented jaundice, mild hypotonia, and a large posterior fontanelle. LT_4 therapy was started at 13 days of age. By 18 days of age, a ^{123}I scintigraphy showed a hypoplastic gland in normal position (Figure 1). The parents

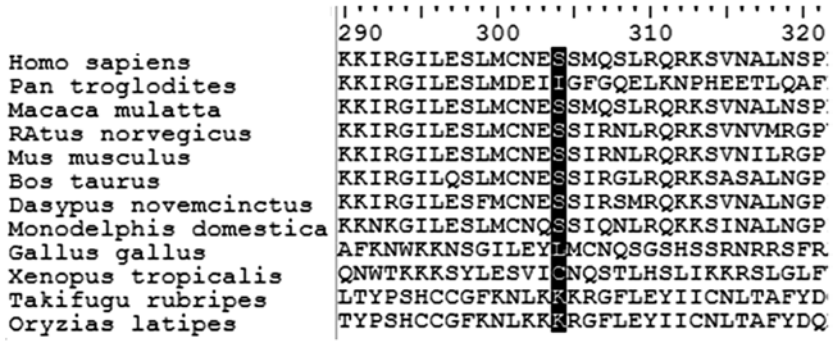

Figure 1 Partial alignment of the human TSHR N-terminal hinge region with amino acid conservation.
Sequencing alignment from different species shows the status of conservation of the mutated amino acid residue. This human TSHR sequence includes 31 amino acid residues located within the HinR of the *TSHR* gene and multiple sequence alignments with 11 orthologs. The shaded boxes indicate the position of the reported p.S304R variant.

were clinically unaffected, but biological investigations showed moderate TSH elevation in the father (TSH, 6.2 μU/mL; normal: 0.4–4.5) with an FT_4 level of 1.0 ng/dL (normal: 0.8–1.8). The propositus' brother had a normal thyroid gland at ultrasound and normal thyroid function tests with a TSH of 2.5 μU/mL (normal: 0.4–4.5). Subsequent analysis of the direct family members revealed that his paternal grandmother had medical history of a mild high TSH of 9.4 μU/mL (normal: 0.4–4.5). By 5 years old, thyroid ultrasound revealed a very hypoplastic thyroid gland. Clinical assessment to date reveals normal growth and development. All family members were studied for mutation in other known candidate genes for thyroid dysgenesis. The patient's father and brother have a normal PAX8 coding sequence, and there were no other *TSHR* sequence variations among the members of this family. The propositus was heterozygous for the previously reported p.R31C PAX8 mutation as previously reported in other studies (31–33). The p.S304R variant was not found in 100 normal individuals.

Ortholog alignment

Our analysis rests on the idea that the differences in standard physicochemical properties between the "wild-type" amino acid and the missense variant are the root cause of functional impairment, and that evolutionary variation among orthologs in the affected position is a sample of the physicochemical properties tolerated at that position. We first built a multiple alignment of orthologs with ClustalW2 (Figure 1) (34); paralogs were excluded to avoid including evolutionary variation that specified functional differences. We have predicted orthologous TSHR protein sequences from the genomic sequences of 11 non-human vertebrate species, including two primates, one artiodactyl, one carnivore, three rodents, one bird, one amphibian and two teleosts species, successfully aligning all to the human reference (Figure 1). The corresponding proteins for each of the abovementioned species were identified and aligned (Figure 1). As expected, maximal identity was observed between the reference (human) and the most recently diverged species, the non-human primates. Similarity decreased as we progressed through the mammalian radiation to greater evolutionary distances, and was at its lowest as we reached the avian and teleost species.

Functional studies of *TSHR*-S304R

Substitution of an arginine for serine 304 modified the consensus sequence of an N-glycosylation site (NXS/T), changing the ^{302}NES^{304} sequence to ^{302}NER^{304}. The glycosylation status of the S304R-mutated *TSHR* gene (S304R-*TSHR*) was determined by Western blot, with an antibody directed against the extracellular domain of TSHR. In protein extracts from cells transiently expressing WT-*TSHR*, this antibody revealed two bands corresponding to the high-mannose form at 95 kDa and to the alpha subunit at 56 kDa (35). After deglycosylation by endoglycosidase F (EndoF), which removed all complex sugars from the N-glycosylation site, two bands appeared, at 80 and 37 kDa, respectively (Figure 2B). In membrane proteins extracted from HEK293 cells transiently expressing S304R-*TSHR*, two bands were also detected, but their molecular weights were slightly lower than those observed for WT-*TSHR* (Figure 2B). These differences between S304R-*TSHR* and WT-*TSHR* disappeared after EndoF treatment. Therefore, the S304R substitution

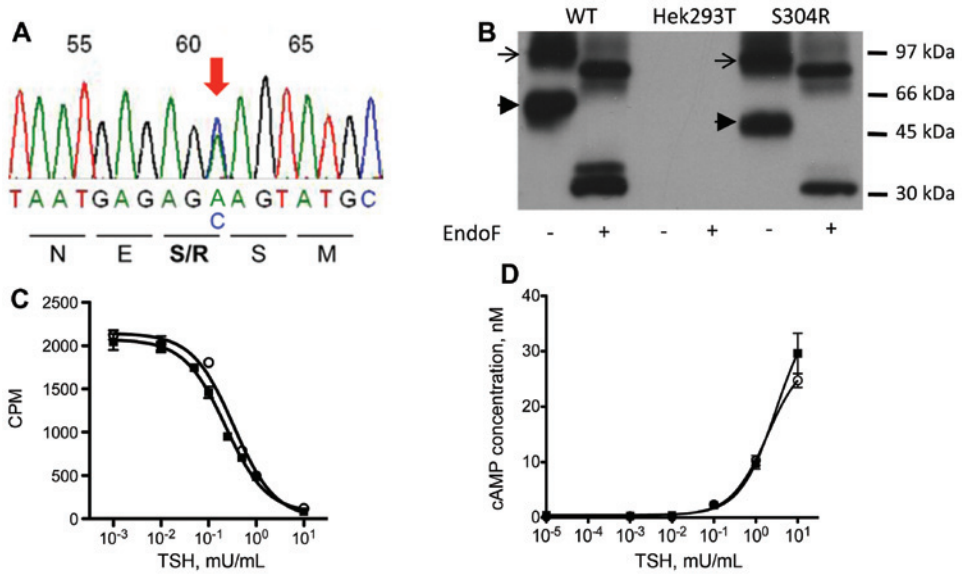

Figure 2 Functional analysis of the S304R-*TSHR* variant in transiently transfected HEK293 cells.
(A) Chromatograms showing the heterozygous p.S304R *TSHR* variant found in family 2. (B) Analysis of the glycosylation profile of WT-*TSHR* and S304R-*TSHR* by Western immunoblotting with and without EndoF treatment. The high-mannose immature form (arrow) and the alpha subunit (arrowhead) of *TSHR* are shown. (C) I^{125}-TSH competitive binding curve with intact cells transiently expressing WT-*TSHR* (black square) or S304R-*TSHR* (open circle). (D) cAMP production in HEK293 transiently expressing WT-*TSHR* (black square) or S304R-*TSHR* (open circle). Each point represents the mean±SEM of hexaplicates.

disrupted N-glycosylation of asparagine 302 (Figure 2B). Functional analyses showed that the S304R substitution affected neither TSH binding to TSHR nor adenylate-cyclase pathway activation, thus indicating that neither N-glycosylation of N302 nor ^{302}NES^{304} sequence was critical for *TSHR* function (Figures 2C and 2D).

Discussion

In a CH patient, we identified a novel heterozygous germline point mutation, at codon 304, which resulted in the substitution of a serine for an arginine (p.S304R) of the TSHR. The mutant receptor was transiently expressed in HEK293 cells and caused equivalent activation of the cAMP pathway when compared with the WT TSHR (Figure 2). The p.S304R variant was identified in only one of the 440 chromosomes analyzed in this cross-sectional study and control population. Therefore, this variation is not considered polymorphism but may be considered a rare sequence variant instead. The analysis of amino acid residue serine in the position 304 of the protein of the *TSHR* gene showed conservation among *Homo sapiens*, *Macaca mulatta, Rattus norvegicus, Mus musculus, Bos taurus, Dasypus novemcinctus* and *Monodelphis domestica* (Figure 1). However, although mutational analyses may be significantly enhanced by deep evolutionary sequence comparisons, they may not account for all sources of functional constraint and phenotypic impact.

The TSHR ectodomain can be subdivided into leucine-rich repeat (LRR) and the HinR, which links the LRR with the serpentine domain and is assumed to be represented by amino acids placed between positions 280 and 410, which contain multiple possible TSH binding sites (9). Therefore, it was likely that the change of a polar hydrophilic S304 into a positive charged arginine residue disrupted the HinR structure, leading to a reduction in the receptor binding capacity. The p.S304R variant is located in a glycosilation HinR position within the onset of the of the cysteine box 2/3 linker region, which is known to be responsible to connect the N- and C-terminally located cysteine boxes [cysteine box 2 (C-b2) and Cysteine box 3 (C-b3)] (36). The value of the C-b2/3 linker in the maintenance of the signaling competence of the receptor has been revealed by several studies (5, 36). More specifically, the role for the serine in the 304 position has been explored and its substitution for alanine (p.S304A mutant) had 32% reduction in the expression and a slight but significant impairment (15%) in binding both bovine TSH (bTSH) and TR1401 (a human TSH analog) (10). Nevertheless, another study confirmed that p.S304A had decreased cell surface expression and transfection efficiency by FACS analysis,

but the basal and stimulated cAMP accumulation were normal compared with WT receptor (36).

Actually, the cysteine box 2/3 linker region and C-terminal portion of HinR harbor other previously described, naturally occurring inactivating mutations (p.R310C, p24X, p.C390W, p.D403N, and p.D410N), and site-directed mutagenesis studies have demonstrated that even the replacement of a single amino acid residue (e.g., p24X, p.C390W, and p.D403N) can severely impair the expression of a functional receptor protein (26, 37–43) (Table 2). Most described patients with a TSHR mutation within the HinR have a thyroid of reduced size, suggesting that this region plays an important role in the signal transduction and, consequently, in the thyroid gland growth (Table 2) (26, 37–45, 47).

Meanwhile, the mutations p.R310C and p.C390W are related with impaired binding and cAMP generation, confirming the hypothesized inhibitory effect of the extracellular domain on a noisy transmembrane; this also explains the compensated TSH resistance in affected patients. On the one hand, the mutation D410N has been reported to be associated with normal binding but defective cAMP generation, and such findings confirm the role of the HinR in conveying the signal from the extracellular domain to the serpentine domain. On the other hand, our functional analysis could express cDNA for the WT and the p.S304R receptor variant in HEK293 cells. The ^{125}I-thyrotropin binding capacity analysis also showed that both forms exhibited similar specific and saturable binding of thyrotropin and the amount of expressed cells on the surface (Figure 2B–D). Further, the aditional functional characterization of the *TSHR*-S304R variant showed that the absence of one N-glycosylation site did not modify receptor function (Figure 2B). In this context, the p.S304R hinge variant does not seem to be functionally important, and the described dysgenetic phenotype would only be explained by the heterozygous p.R31C mutation, which was exclusively found in the propositus patient. The deleterious effect of PAX8 mutation has already been demonstrated (33). We can assume that the wearing 2 mutations on the PAX8 and TSHR synergize default size and functional thyroid.

Understanding the structure and function of the HinR is an important step in achieving a complete picture of the

Table 2 Spectrum of clinical features and functional effects in patients with *TSHR* gene missense mutations located at the hinge portion identified to date.

Protein	No. of cases	Genotype	Thyroid morphology	Thyroid function	Functional analysis of the mutations	References
S304R	1	HET	Hypoplasia	CH	nl cAMP nl Glycosilation	Present study
R310C	5[a]	3 HET	nl	MH	↓ TSH Binding	(44)
		2 HOM		SH	↓ cAMP	
	1	HET	Nl	MH		(40)
C390W	1	HET	enlarged	MH	↓ Expression at cell surface	(26)
	3[a]	2 HET	nl	MH	↓ TSH binding ↓ cAMP	(40)
	4[a]	3HET	nl	EU		
		1HOM	Hypoplasia	CH		(36)
	1	HET	na	MH		(37)
D403N	1	HET	na	MH	↓ TSH binding	(38)
	2[a]	HET	na	EU, IH	↓ Expression at cell surface ↓ cAMP	(39)
	2[a]	HET	Hypoplasia	MH, IH		(40)
	1	HET	na	IH		(41)
	1	CHET	na	CH		(43)
D410N	2[a]	HET	nl	MH, IH	=TSH Binding ↓ cAMP	(40)
	1	CHET	nl	MH		(26)
	2[a]	HET	nl	IH		(45)

Adapted from Cassio et al. (12) and Persani et al. (46). EU, euthyroidism; IH, isolated hyperthyrotropinemia; MH, mild hypothyroidism; SH, severe hypothyroidism; CH, congenital hypothyroidism; HOM, homozygous; HET, heterozygous; CHET, compound heterozygous. [a]Member of the same family. na, not given; nl, within normal limits.

necessary mechanisms for the TSHR activation and signaling. Recent reports have shown that the HinR is not only a scaffold for maintaining binding of the glycoprotein hormones, but may also work as an important signal transmitter for TSH-induced activation.

Conflict of interest statement: The authors have no conflict of interest to disclose. Informed consent has been obtained from the patient (or patient's guardian) for publication of the case report.

Financial support: This work was supported in part by grants from Electricité de France (RB 200605), European Society for Pediatric Endocrinology (ESPE) Research Unit (MP), *Fondation Grace de Monaco* and PHRC 2011 Hypotygen sponsored by the French Ministry, Fundação de Amparo à Pesquisa no Estado da Bahia (FAPESB) (HER), Société française d'endocrinologie (SFE), MercK Serono Grant (MC), and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (HER). TLOC obtained financial support from CAPES.

References

1. Vassart G, Pardo L, Costagliola S. A molecular dissection of the glycoprotein hormone receptors. Trends Biochem Sci 2004;29:119–26.
2. Duprez L, Parma J, Van Sande J, Rodien P, Sabine C, et al. Pathology of the TSH receptor. J Pediatr Endocrinol Metab 1999;12:295–302.
3. Vassart G, Desarnaud F, Duprez L, Eggerickx D, Labbe O, et al. The G protein-coupled receptor family and one of its members, the TSH receptor. Ann N Y Acad Sci 1995;766:23–30.
4. Tonacchera M, Van Sande J, Parma J, Duprez L, Cetani F, et al. TSH receptor and disease. Clin Endocrinol (Oxf) 1996;44: 621–33.
5. Vlaeminck-Guillem V, Ho SC, Rodien P, Vassart G, Costagliola S. Activation of the cAMP pathway by the TSH receptor involves switching of the ectodomain from a tethered inverse agonist to an agonist. Mol Endocrinol 2002;16:736–46.
6. Duprez L, Parma J, Costagliola S, Hermans J, Van Sande J, et al. Constitutive activation of the TSH receptor by spontaneous mutations affecting the N-terminal extracellular domain. FEBS Lett 1997;409:469–74.
7. Hamidi S, Chen CR, Mizutori-Sasai Y, McLachlan SM, Rapoport B. Relationship between thyrotropin receptor hinge region proteolytic posttranslational modification and receptor physiological function. Mol Endocrinol 2011;25:184–94.
8. Jaeschke H, Schaarschmidt J, Gunther R, Mueller S. The hinge region of the TSH receptor stabilizes ligand binding and determines different signaling profiles of human and bovine TSH. Endocrinology 2011;152:3986–96.
9. Mueller S, Jaeschke H, Gunther R, Paschke R. The hinge region: an important receptor component for GPHR function. Trends Endocrin Met 2009;21:111–22.
10. Mueller S, Szkudlinski MW, Schaarschmidt J, Gunther R, Paschke R, et al. Identification of novel TSH interaction sites by systematic binding analysis of the TSHR hinge region. Endocrinology 2011;152:3268–78.
11. Smits G, Govaerts C, Nubourgh I, Pardo L, Vassart G, et al. Lysine 183 and glutamic acid 157 of the TSH receptor: two interacting residues with a key role in determining specificity toward TSH and human CG. Mol Endocrinol 2002;16:722–35.
12. Persani L, Calebiro D, Cordella D, Weber G, Gelmini G, et al. Genetics and phenomics of hypothyroidism due to TSH resistance. Mol Cell Endocrinol 2010;322:72–82.
13. Sunthornthepvarakui T, Gottschalk ME, Hayashi Y, Refetoff S. Brief report: resistance to thyrotropin caused by mutations in the thyrotropin-receptor gene. N Engl J Med 1995;332:155–60.
14. Calebiro D, de Filippis T, Lucchi S, Covino C, Panigone S, et al. Intracellular entrapment of wild-type TSH receptor by oligomerization with mutants linked to dominant TSH resistance. Hum Mol Genet 2005;14:2991–3002.
15. Calebiro D, Gelmini G, Cordella D, Bonomi M, Winkler F, et al. Frequent TSH receptor genetic alterations with variable signaling impairment in a large series of children with nonautoimmune isolated hyperthyrotropinemia. J Clin Endocrinol Metab 2012;97:E156–60.
16. Polak M, Sura-Trueba S, Chauty A, Szinnai G, Carre A, et al. Molecular mechanisms of thyroid dysgenesis. Horm Res 2004;62:14–21.
17. Sunthornthepvarakul T, Hayashi Y, Refetoff S. Polymorphism of a variant human thyrotropin receptor (hTSHR) gene. Thyroid 1994;4:147–49.
18. Gustavsson B, Eklof C, Westermark K, Westermark B, Heldin NE. Functional analysis of a variant of the thyrotropin receptor gene in a family with Graves' disease. Mol Cell Endocrinol 1995;111:167–73.
19. Gabriel EM, Bergert ER, Grant CS, van Heerden JA, Thompson GB, et al. Germline polymorphism of codon 727 of human thyroid-stimulating hormone receptor is associated with toxic multinodular goiter. J Clin Endocrinol Metab 1999;84:3328–35.
20. Peeters RP, van Toor H, Klootwijk W, de Rijke YB, Kuiper GG, et al. Polymorphisms in thyroid hormone pathway genes are associated with plasma TSH and iodothyronine levels in healthy subjects. J Clin Endocrinol Metab 2003;88:2880–88.
21. Hansen PS, van der Deure WM, Peeters RP, Iachine I, Fenger M, et al. The impact of a TSH receptor gene polymorphism on thyroid-related phenotypes in a healthy Danish twin population. Clin Endocrinol (Oxf) 2007;66:827–32.
22. Nogueira CR, Kopp P, Arseven OK, Santos CL, Jameson JL, et al. Thyrotropin receptor mutations in hyperfunctioning thyroid adenomas from Brazil. Thyroid 1999;9:1063–68.
23. Sykiotis GP, Neumann S, Georgopoulos NA, Sgourou A, Papachatzopoulou A, et al. Functional significance of the thyrotropin receptor germline polymorphism D727E. Biochem Biophys Res Commun 2003;301:1051–56.
24. Cuddihy RM, Bryant WP, Bahn RS. Normal function in vivo of a homozygotic polymorphism in the human thyrotropin receptor. Thyroid 1995;5:255–57.
25. Loos U, Hagner S, Bohr UR, Bogatkewitsch GS, Jakobs KH, et al. Enhanced cAMP accumulation by the human thyrotropin receptor variant with the Pro52Thr substitution in the extracellular domain. Eur J Biochem 1995;232:62–65.

26. de Roux N, Misrahi M, Brauner R, Houang M, Carel JC, et al. Four families with loss of function mutations of the thyrotropin receptor. J Clin Endocrinol Metab 1996;81:4229–35.
27. Karlin S, Mrazek J, Gentles AJ. Genome comparisons and analysis. Curr Opin Struct Biol 2003;13:344–52.
28. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol 1990;215:403–10.
29. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 1994;22: 4673–80.
30. Sura-Trueba S, Aumas C, Carre A, Durif S, Leger J, et al. An inactivating mutation within the first extracellular loop of the thyrotropin receptor impedes normal posttranslational maturation of the extracellular domain. Endocrinology 2009;150:1043–50.
31. Ramos HE, Gabor Szinnai AC, Tron E, Cerqueira TL, Leger J, et al. PAX8 Mutations in congenital hypothyroidism: New evidence for phenotypic variability from normal to ectopic thyroid gland. Eur J Endocrinol 2014, in Press. DOI: http://dx.doi.org/10.1530/EJE-13-1006.
32. Komatsu M, Takahashi T, Takahashi I, Nakamura M, Takahashi I, et al. Thyroid dysgenesis caused by PAX8 mutation: the hypermutability with CpG dinucleotides at codon 31. J Pediatr 2001;139:597–99.
33. Ramos HE, Labedan I, Carre A, Castanet M, Guemas I, et al. New cases of isolated congenital central hypothyroidism due to homozygous thyrotropin beta gene mutations: a pitfall to neonatal screening. Thyroid 2010;20:639–45.
34. Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, et al. Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res 2003;31:3497–500.
35. Trueba SS, Auge J, Mattei G, Etchevers H, Martinovic J, et al. PAX8, TITF1, and FOXE1 gene expression patterns during human development: new insights into human thyroid development and thyroid dysgenesis–associated malformations. J Clin Endocrinol Metab 2005;90:455–62.
36. Kleinau G, Mueller S, Jaeschke H, Grzesik P, Neumann S, et al. Defining structural and functional dimensions of the extracellular thyrotropin receptor region. J Biol Chem 2011;286:22622–31.
37. Biebermann H, Schoneberg T, Krude H, Schultz G, Gudermann T, et al. Mutations of the human thyrotropin receptor gene causing thyroid hypoplasia and persistent congenital hypothyroidism. J Clin Endocrinol Metab 1997;82:3471–80.
38. Calaciura F, Motta RM, Miscio G, Fichera G, Leonardi D, et al. Subclinical hypothyroidism in early childhood: a frequent outcome of transient neonatal hyperthyrotropinemia. J Clin Endocrinol Metab 2002;87:3209–214.
39. Camilot M, Teofoli F, Gandini A, Franceschi R, Rapa A, et al. Thyrotropin receptor gene mutations and TSH resistance: variable expressivity in the heterozygotes. Clin Endocrinol (Oxf) 2005;63:146–51.
40. De Marco G, Agretti P, Camilot M, Teofoli F, Tato L, et al. Functional studies of new TSH receptor (TSHr) mutations identified in patients affected by hypothyroidism or isolated hyperthyrotrophinaemia. Clin Endocrinol (Oxf) 2009;70:335–38.
41. Nicoletti A, Bal M, De Marco G, Baldazzi L, Agretti P, et al. Thyrotropin-stimulating hormone receptor gene analysis in pediatric patients with non-autoimmune subclinical hypothyroidism. J Clin Endocrinol Metab 2009;94:4187–94.
42. Rapa A, Monzani A, Moia S, Vivenza D, Bellone S, et al. Subclinical hypothyroidism in children and adolescents: a wide range of clinical, biochemical, and genetic factors involved. J Clin Endocrinol Metab 2009;94:2414–20.
43. Tonacchera M, Agretti P, De Marco G, Perri A, Pinchera A, et al. Thyroid resistance to TSH complicated by autoimmune thyroiditis. J Clin Endocrinol Metab 2001;86:4543–46.
44. Narumi S, Muroya K, Abe Y, Yasui M, Asakura Y, et al. TSHR mutations as a cause of congenital hypothyroidism in Japan: a population-based genetic epidemiology study. J Clin Endocrinol Metab 2009;94:1317–23.
45. Russo D, Betterle C, Arturi F, Chiefari E, Girelli ME, et al. A novel mutation in the thyrotropin (TSH) receptor gene causing loss of TSH binding but constitutive receptor activation in a family with resistance to TSH. J Clin Endocrinol Metab 2000;85:4238–42.
46. Cassio A, Nicoletti A, Rizzello A, Zazzetta E, Bal M, et al. Current loss-of-function mutations in the thyrotropin receptor gene: when to investigate, clinical effects, and treatment. J Clin Res Pediatr Endocrinol 2013;5:29–39.
47. Tonacchera M, Di Cosmo C, De Marco G, Agretti P, Banco M, et al. Identification of TSH receptor mutations in three families with resistance to TSH. Clin Endocrinol (Oxf) 2007;67:712–18.