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Case History

A Novel Activating Mutation in Transmembrane Helix 6 of the Thyrotropin Receptor as Cause of Hereditary Nonautoimmune Hyperthyroidism

Benjamin U. Nwosu,¹ Loukas Gourgiotis,² Marvin C. Gershengorn,² and Susanne Neumann²

Constitutively-activating germline mutations of the thyrotropin receptor (TSHR) gene are very rare and are considered the cause of hereditary nonautoimmune hyperthyroidism. We describe four affected individuals from a Caucasian family: a mother and her three children, and an unaffected father. The mother and her first two children presented in a similar manner: lifelong histories of heat intolerance, hyperactivity, fast heart rate, reduced energy, increased appetite, and scrawny build. They all developed goiter in childhood and showed a suppressed TSH and elevated thyroxine (T₄). The last child, a 12-year-old female, presented with no clinical symptoms or palpable neck mass, but with a suppressed TSH, elevated T₄ and thyromegaly detected by ultrasound. Mutation analysis of the TSHR gene in all family members revealed a novel heterozygous germline mutation resulting in the substitution of phenylalanine (TTC) by serine (TCC) at codon 631 in transmembrane helix 6 in the mother and all three children. Functional characterization of this germline mutation showed constitutive activation of the G_s-mediated cyclic adenosine monophosphate (cAMP) pathway, which controls thyroid hormone production and thyroid growth. Molecular characterization of F631S demonstrates that this activating mutation plays a key role in the development of hereditary hyperthyroidism in this family although the timing of onset of clinical manifestations in the subjects may depend on other, as yet unidentified, factors.

Introduction

THE THYROTROPIN RECEPTOR (TSHR) belongs to the superfamily of seven transmembrane-spanning receptors, specifically to the subfamily of glycoprotein hormone receptors. Binding of the ligand, thyrotropin (TSH), to its receptor leads to activation of the G_{αs}-mediated cyclic adenosine monophosphate (cAMP) pathway and at higher concentrations of the G_{αq}-mediated phospholipase C-inositol phosphate (IP) pathway (1). As activation of the phospholipase C cascade requires 10 times higher TSH concentrations, it is generally accepted that cAMP activation is the major pathway that controls growth and function of thyrocytes (2,3). The phospholipase C pathway is involved in control of iodination and thyroid hormone synthesis (4).

Numerous gain-of-function mutations of TSHR that cause constitutive (ligand-independent) activation of the cAMP cascade have been identified in different thyroid patholo-

gies (2,3,5) (www.uni-leipzig.de/~innere/tsh/). For a few mutants, constitutive activation of both cAMP and phospholipase C cascades has been reported (6). Somatic activating mutations of TSHR have been identified as the cause of solitary toxic adenomas and multinodular goiter (2,3,6). Somatic mutations also have been reported in thyroid carcinomas, albeit much more infrequently (7–9). Germline activating TSHR mutations cause congenital nonautoimmune hyperthyroidism, which is a rare inherited condition (2,5,10). These heterozygous autosomal dominant germline mutations underline convincingly the causal impact of TSHR mutations for the pathogenesis of hyperthyroidism. The prominent clinical characteristics of nonautoimmune hyperthyroidism are autosomal dominant transmission, a positive family history of hyperthyroidism, and hyperplastic goiter with an early occurrence of the disease and recurrence after medical treatment (11). The age of onset of hyperthyroidism varies, which may be explained by differ-

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ences in environmental factors, in particular iodine intake, or might be the result of other genetic components. Commonly, familial nonautoimmune hyperthyroidism is confused with Graves' disease, an autoimmune disorder caused by activating TSHR antibodies (12,13). However, familial occurrence of hyperthyroidism in the absence of TSHR antibodies is highly suggestive of familial nonautoimmune hyperthyroidism resulting from a constitutively activating TSHR germline mutation. Thyroid ablation (surgery and/or radioiodine) is recommended as therapy because there is no likelihood of spontaneous remission of hyperthyroidism (2). Detection and molecular analysis of germline mutations in families with nonautoimmune hyperthyroidism has an important clinical impact with respect to genetic counseling and presymptomatic diagnosis of hyperthyroidism in children from affected families.

The first case of nonautoimmune hyperthyroidism was described in 1982 in the Nancy family in which 9 of the 34 members studied were found to be thyrotoxic with a diffusely enlarged goiter and no signs of Graves' disease or presence of serum autoantibodies (14). Molecular analysis revealed a heterozygous germline mutation that leads to a valine to alanine substitution at amino acid position 509 in transmembrane helix (TMH) 3 of TSHR (10). Subsequently, 19 germline TSHR mutations in 12 families and 7 children with sporadic occurrence have been reported (5) (www.uni-leipzig.de/~innere/tsh/). Interestingly, the majority of germline mutations (63%) is not observed in toxic thyroid nodules. This is compatible with the observation that hereditary mutations cause a less severely affected phenotype, with a marginal effect on reproductive fitness (2,10,15,16). Because of limited cases of germline mutations, no systematic study has yet been performed to demonstrate a correlation between genotype and phenotype. Such an approach might also be limited in its conclusions due to the influence of environmental and other genetic factors. Nevertheless, identification and molecular characterization of naturally occurring mutants has led to significant progress in diagnosis of nonautoimmune hyperthyroidism and in our understanding of the TSHR activation mechanism.

In this study, we report a novel activating TSHR germline mutation at amino acid position 631 in TMH6 in a mother and her three children, all affected by hyperthyroidism (Fig. 1A). Subsequent functional *in vitro* characterization of the mutant F631S revealed constitutive activation of the cAMP regulatory cascade demonstrating that this mutation plays a key role in development of hereditary hyperthyroidism in this family.

Case Report

The proband is a 47-year-old Caucasian female who has had lifelong symptoms suggestive of hyperthyroidism. She had been born to a 30-year-old G3 P2 female. Her birth weight was 2200 g. She had no perinatal complications and achieved normal milestones for age. She recalls that as a youngster she had always felt that she was "burning up on the inside" and describes herself as being scrawny, sweaty, with a fast heart rate, and hyperactive demeanor in association with reduced energy, increased appetite, and intolerance to heat. Her thyroid gland progressively increased in size. At age 42 years, she presented to a tertiary care hospi-

tal with a neck mass and prominent eyes. Her physical examination revealed proptosis, goiter, smooth skin, mild tremor of the extremities and brisk deep tendon reflexes. Her thyroid function test (TFT) results were consistent with hyperthyroidism (Table 1). Her radioactive iodine uptake was 78% (normal, 10%–35%) in 24 hours. A radioactive iodine scan revealed diffuse uptake throughout both lobes of a mildly enlarged gland with a firm smooth hypofunctioning nodule in the region of the isthmus. She had a fine-needle aspiration biopsy (FNAB) of the nodule, which was consistent with a benign colloid nodule. She was diagnosed with Graves' disease and placed on methimazole, which she tolerated poorly. Subsequently, she had radioactive iodine ablation with 6 mCi of ¹³¹I. Five months later, her TFTs normalized (Table 1). She was started on levothyroxine 50 µg daily. Despite normalization of serum thyroxine levels, she complained of fatigue, muscle aches, and inability to lose weight. Levotriiodothyronine was added empirically and she was maintained on levotriiodothyronine 10 µg and levothyroxine 75 µg daily.

The patient's family history revealed that she is one of three children. She says that both her father and paternal grandmother had "bug eyes," but they were never evaluated for any endocrine dysfunction. She has two brothers who appear normal. She is married and has three children. Her first child, a 24-year-old male, was diagnosed with hyperthyroidism at 19 years of age. He had similar lifelong symptoms as his mother but no visible neck mass. He was born at 34 weeks of gestation. He weighed 2200 g and was 50.8 cm long. His TFTs are presented in Table 1. His neck ultrasound showed an enlarged thyroid gland primarily in the anteroposterior and transverse directions. The right lobe measured 4.8 × 2.0 × 2.2 cm, and the left lobe 5.3 × 2.3 × 2.6 cm. Thyroid parenchyma was diffusely patchy and micronodular in echotexture, with scattered millimeter-sized nonspecific appearing nodules in both lobes of the thyroid gland. He was started on methimazole 10 mg three times daily. Three months later, he normalized his T₄ level but TSH remained suppressed at less than 0.05 µU/mL.

The proband's second child is an 18-year-old daughter who was diagnosed with hyperthyroidism at 11 years of age. She was born at 34 weeks of gestation. Her birth weight was 2130 g and she was 50.8 cm long. She has had similar lifelong symptoms as her mother and brother. Her TFTs are presented in Table 1. She had an enlarged thyroid gland with a neck ultrasound revealing a multinodular goiter with several dominant-sized complex nodules in both lobes. The right lobe measured 8.5 × 2.4 × 3.7 cm, and the left lobe measures 8.2 × 2.7 × 3.1 cm. A multiloculated nodule, measuring 3.0 × 1.5 × 2.0 cm with internal solid components, was identified in the lower pole of the left lobe. Radioactive iodine scan showed a uniform bilobar uptake with the exception of two small nodules, one in each lobe. Her 6-hour thyroid uptake was 100%. She was started on methimazole, which was discontinued because of side effects.

The proband's last child is a 12-year-old daughter with cerebral palsy who was diagnosed with hyperthyroidism at 10 years of age during a follow-up. She was born at 30 weeks of gestation. She weighed 1310 g, and measured 43.2 cm. She has neither a goiter nor any of the hyperthyroid symptoms shown by her mother and siblings. Her physical examination revealed a wide pulse pressure, smooth skin, and

A

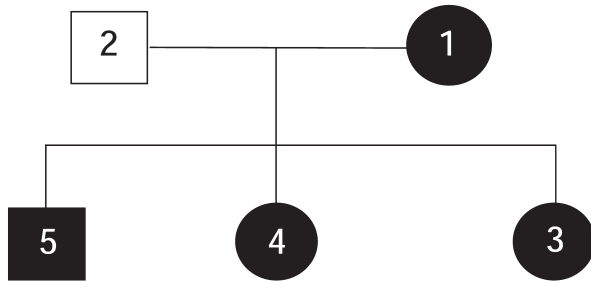
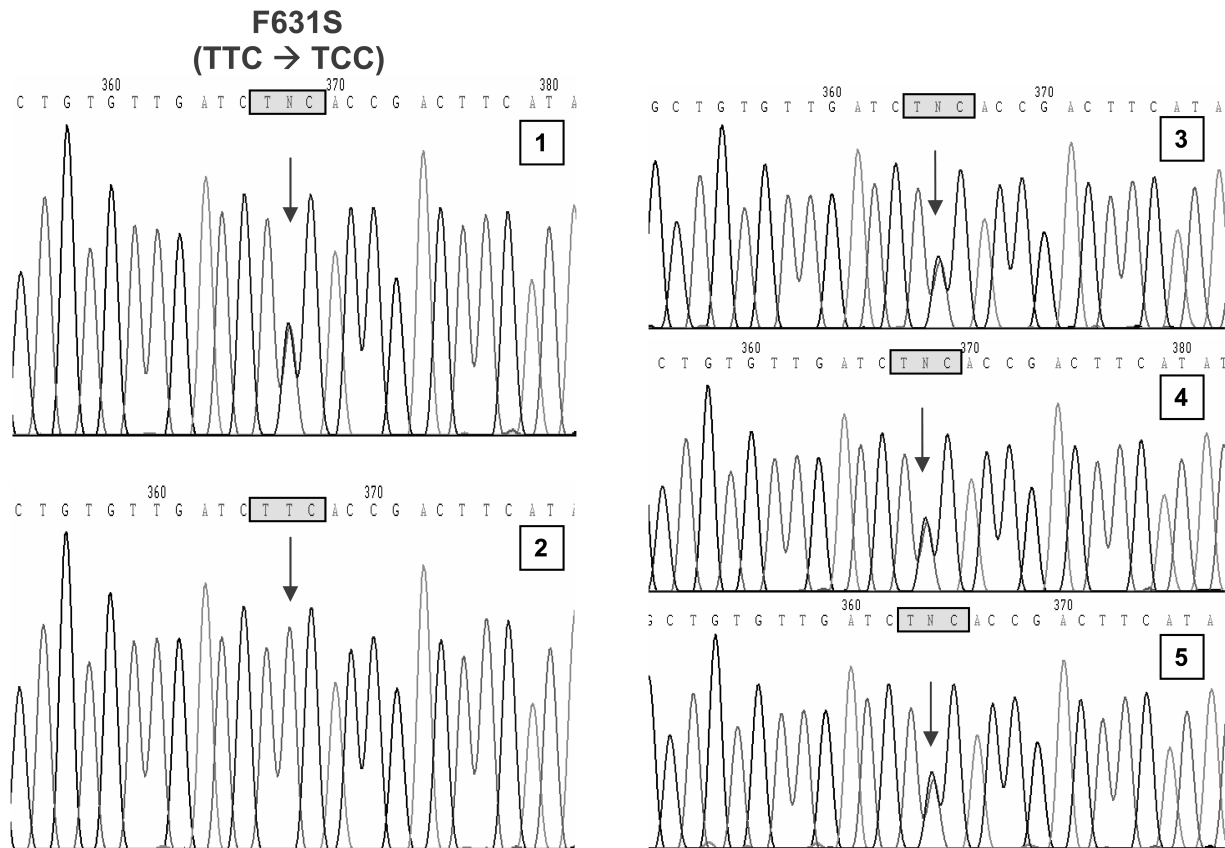


FIG. 1. Analysis of the TSHR gene in all family members. **A:** Pedigree of the family. All affected family members are indicated by filled symbols. **B:** Sequencing of one polymerase chain reaction (PCR) fragment of exon 10 revealed an identical heterozygous TSHR mutation in the children and their mother at nucleotide position 1892, where a T is substituted by a C, resulting in substitution of phenylalanine (TTC) with serine (TCC) at residue 631 of the TSHR. The numbering of the pedigree symbols and the sequences correspond to each other.

B



slightly exaggerated tendon reflexes. Her TFTs are presented in Table 1. Her neck ultrasound showed a normal thyroid gland size and contour with no nodularity. She was started on 5 mg methimazole daily. She complained of muscle aches, pains, and weight gain, necessitating the reduction of her dose to 2.5 mg every other day. TSHR antibodies were negative in the proband and all three children. The proband had positive antithyroidal antibodies (anti-Tg 200 IU/mL [normal, < 40], anti-TPO 105 IU/mL [normal, < 35]).

The study was approved by the Institutional Review Board of the Intramural Division of the National Institute of Diabetes and Digestive and Kidney Diseases and informed consent was obtained from all family members.

Materials and Methods

DNA analysis of the TSHR gene

Because of the familial occurrence of hyperthyroidism, blood samples were taken from the parents and the three children. Genomic DNA was extracted from the patients' peripheral blood leukocytes using the QIAamp DNA Blood Midi Kit (QIAGEN, Valencia, CA). Two overlapping fragments of exon 10 of the TSHR were amplified by polymerase chain reaction (PCR) using YieldAce Hotstart DNA polymerase (Stratagene, La Jolla, CA) according to the instructions of the manufacturer. We used PCR primers for exon 10 published by De Roux et al. (17). The PCR products were

TABLE 1. HORMONE STATUS, DURATION OF PREGNANCY, AND BIRTH WEIGHT DUE TO AUTOSOMAL-DOMINANT NONAUTOIMMUNE HYPERTHYROIDISM

	TSH (NR 0.28– 3.89 $\mu\text{U/mL}$)	Total T ₄ (NR 4.5–12.5 $\mu\text{g/dL}$)	Free T ₄ (NR 0.58–1.64 ng/dL)	Total T ₃ (NR 80–213 ng/dL)	Pregnancy duration (weeks) ^a	Birth weight (g)
Mother (before RAI)	0.04	20.63		434		2200
Mother (post-RAI)	0.41		0.79			
Son	0.05	21.70			34	2200
First daughter	0.01	14.20	5.32	592	34	2130
Second daughter	0.04	19.10	2.32	418	30	1310

^aPremature birth: 37 weeks or less; normal range of birth weight: 2720–4530 g; RAI, radioactive iodine; TSH, thyrotropin; T₃, triiodothyronine; T₄, thyroxine.

purified using the MinElute PCR Purification kit (QIAGEN, Valencia, CA) and sequenced using the PCR primers. Sequencing reactions were performed by MWG Biotech, Inc. (High Point, NC).

Site-directed mutagenesis

Human TSHR cDNA in expression vector pcDNA3.1 was obtained from the UMR cDNA Resource Center (www.cdna.org) and used as template for mutagenesis. The F631S mutant was generated using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene) according to the instructions of the manufacturer. Sequence of mutated TSHR was verified by dideoxy sequencing.

Cell culture and transfection

HEK (human embryonic kidney) 293 EM cells (18) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 100 U/mL penicillin, and 10 $\mu\text{g/mL}$ streptomycin (Life Technologies Inc.) at 37°C in a humidified 5% CO₂ incubator. One day before transfection, the cells were seeded in 24-well dishes (0.5×10^5 cells per well). After 24 hours, cells were transiently transfected with 0.2 μg receptor expression vector per well using FuGENE™6 reagent (Roche, Basel, Switzerland). Mock transfections were performed with empty expression vector pcDNA3.1. All assays were performed 48 hours after transfection.

Determination of cell surface expression by FACS analysis

After transfection, cells were cultured for 48 hours, harvested using 1 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM ethyleneglycoltetraacetic acid (EGTA) in phosphate-buffered saline (PBS) and transferred to Falcon 2058 tubes. Cells were washed once with PBS containing 0.1% bovine serum albumin (BSA) and 0.1% NaN₃ (binding buffer), incubated for 1 hour with a 1:200 dilution of mouse anti-human TSHR antibody (Serotec, MCA1281, Clone number 2C11) in binding buffer, washed twice, and incubated for 1 hour in the dark with a 1:200 dilution in binding buffer of an Alexa Fluor 488-labeled F(ab')₂ fragment of goat anti-mouse IgG (Molecular Probes, Eugene, OR). Before FACS analysis (FACS Calibur, BD Biosciences, San Jose, CA), cells

were washed twice and fixed with 1% paraformaldehyde. Receptor expression was estimated by fluorescence intensity and transfection efficiency was measured as the percentage of fluorescent cells.

cAMP accumulation assay

Measurement of cAMP accumulation was performed 48 hours after transfection of HEK 293 cells using a cAMP Biotrak Enzymeimmunoassay according to the instructions of the manufacturer (Amersham Biosciences, Piscataway, NJ).

Measurement of IP formation

Transfected HEK 293 cells were incubated with 2 $\mu\text{Ci/mL}$ of [myo-³H] inositol (18.6 Ci/mmol) (Amersham) for 18 hours. Thereafter, cells were preincubated with serum free DMEM containing 10 mM LiCl for 15 minutes. Stimulation with TSH was performed with the same medium supplemented with 100 mU/mL TSH for 1 hour. Basal and TSH-induced increases in intracellular IP levels were determined by anion exchange chromatography as described (19). IP values are expressed as the percentage of radioactivity incorporated from ³[H]-inositolphosphates over the sum of radioactivity incorporated in IPs and phosphatidyl inositols.

Results

Identification of TSHR mutation

Direct sequencing of exon 10 of the TSHR gene of all family members led to identification of a heterozygous T to C transition at nucleotide position 1892. This heterozygous mutation results in the substitution of a highly conserved phenylalanine (TTC) by serine (TCC) at codon 631 (F631S) in TMH6. The mutation was found in the mother and all her 3 children; however, it was absent in their unaffected father (Fig. 1A and B).

Functional characterization of the novel TSHR germline mutation

HEK 293 cells were transiently transfected with the wild type (wt) TSHR and the F631S mutant. Cells surface expression for the F631S mutant was reduced by 60% compared to TSHR (Fig. 2). Basal and TSH-stimulated cAMP and IP production were measured. F631S showed constitutive activa-

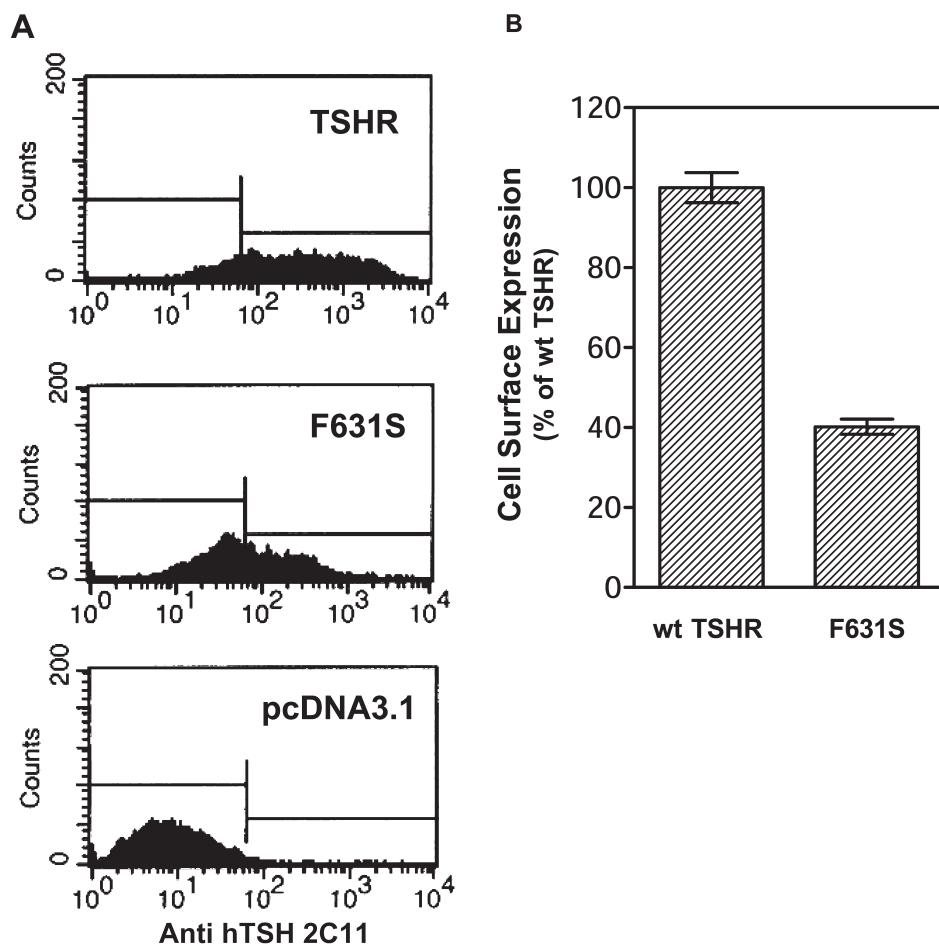


FIG. 2. Cell surface expression of F631S mutant. HEK 293 cells were transiently transfected with pcDNA3.1 vector (control), TSHR or F631S. **A:** Cell surface expression was measured using fluorescence-activated cell sorter (FACS) analysis. Fluorescence intensity is expressed in arbitrary units as function of cell number plotted on logarithmic scale. **B:** The fluorescence signal for the TSHR was set at 100% and the F631S mutant is expressed as percent of TSHR. Cell surface expression is decreased for the F631S mutant compared to TSHR. The data represent the mean \pm standard deviation (SD) of duplicate determinations in two separate experiments.

tion of the G_s -mediated adenylyl cyclase-cAMP pathway. Basal cAMP production of F631S was sevenfold higher than basal TSHR activity (Fig. 3A). TSH-induced cAMP production was not affected by the F631S mutant. In contrast, basal G_q -mediated inositol phosphate formation induced by F631S was comparable to TSHR. However, the TSH-induced IP response was reduced for F631S (30% of maximum TSHR response) (Fig. 3B), which might be partly explained by the low cell surface expression of this mutant.

Discussion

Constitutive activation of TSHR is involved in hyperfunctioning thyroid adenomas and autosomal dominant, nonautoimmune hyperthyroidism caused by heterozygous somatic or germline mutations, respectively. Familial nonautoimmune hyperthyroidism is a rare disorder, with an incidence of less than 1% in patients with juvenile hyperthyroidism (20). Until now, 18 constitutively activating germline mutations of TSHR were reported in cases of hereditary and sporadic nonautoimmune hyperthyroidism (5). All gain-of-function germline mutations were identified in exon 10 encoding the serpentine domain of TSHR with exception of S281N in exon 9 (21), which encodes part of the large ectodomain. One germline mutation at amino acid position-183 in the ectodomain did not lead to constitutive TSHR activation. In this case, hereditary gestational hyperthyroidism was caused by hypersensitivity of the mutated TSHR to

chorionic gonadotropin (22). The majority of germline mutants (44%) has been identified in TMH6 and 7 of TSHR (www.uni-leipzig.de/~innere/tsh/). Herein we report a novel heterozygous germline mutation resulting in substitution of phenylalanine by serine at codon-631 (F631S) in TMH6 of TSHR in the mother and all three children. Constitutive activation of the G_s -mediated cAMP pathway by F631S demonstrates that this novel activating germline mutation plays a key role in the development of hereditary hyperthyroidism in this Caucasian family. Four other amino acid substitutions are known at this highly conserved position: F631I, L, C and V. F631I, V and C have been detected in hyperfunctioning thyroid nodules (23-26). F631L was found in both hyperfunctioning thyroid adenomas and congenital hyperthyroidism (27,28). F631I has not yet been functionally characterized. Mutants F631L, C, and V lead to constitutive activity of TSHR with regard to cAMP production, however, no constitutive activation of the phospholipase C pathway was observed. The novel F631S mutant identified in this family also did not exhibit an increase of basal inositol phosphate production, however, in contrast to the other described mutants, we observed impairment of TSH-induced activation of this pathway (only 30% of maximum TSHR response). This difference among the mutant receptors might be partly explained by diverse helical conformations caused by different mutants at amino acid position 631. However, because all mutants were described in single studies and assays for determination of functional characteristics

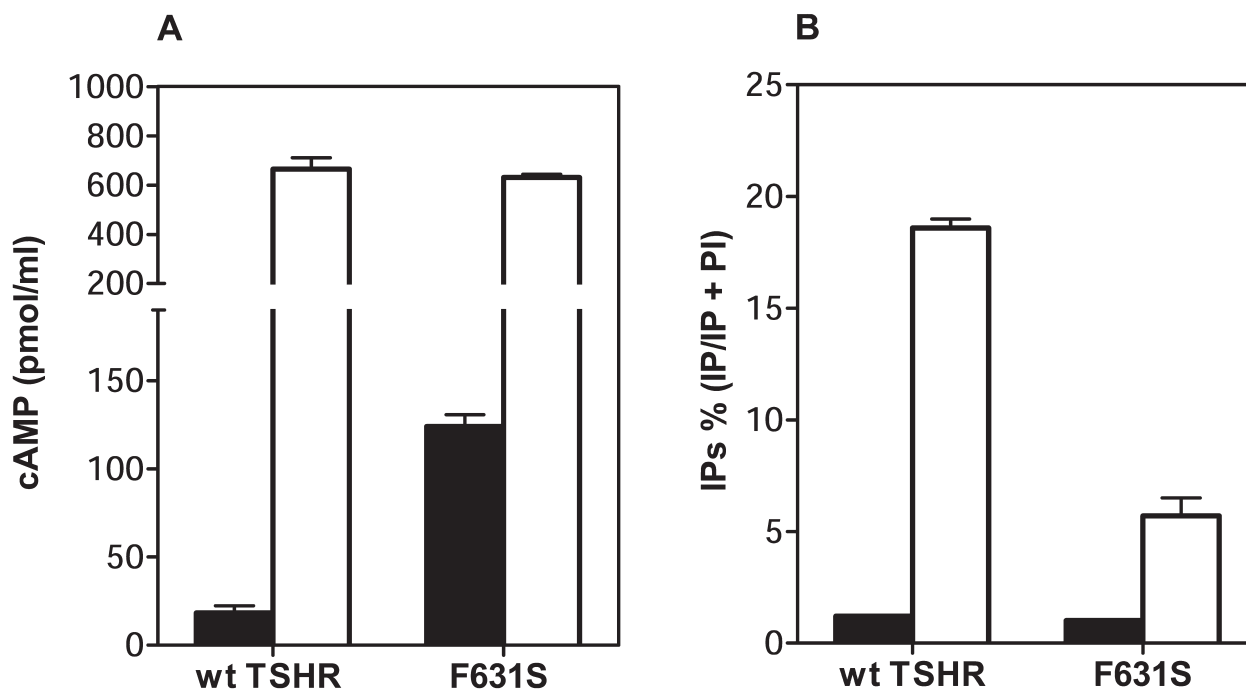


FIG. 3. Functional characterization of F631 mutant. Basal and thyrotropin (TSH)-stimulated cyclic adenosine monophosphate (cAMP; **A**) and inositol phosphate (IP; **B**) accumulation in HEK 293 cells expressing TSHR or F631S in the absence (filled bars) or presence (open bars) of 100 mU/mL bovine TSH. The cAMP data represent the mean \pm standard deviation (SD) of triplicate determinations in one representative experiment out of three separate experiments. IP data represent the mean \pm range of duplicate determinations in 1 representative experiment of 3 separate experiments.

vary, only a direct comparison of all described mutants in one study might allow precise conclusions with regard to pharmacologic and structural differences.

The strong reduction in cell surface expression by F631S in comparison to TSHR could be the result of abnormal folding of the receptor supporting the idea of changes in helical conformation. It has been shown that a mutated constitutively active TSHR or angiotensin II AT (1A) receptor exhibited increased internalization in absence of the ligand (29,30). Moreover, constitutive downregulation and desensitization resulting in low expression levels was demonstrated for several 7TMRs (31). These might be alternative explanations for the reduced cell surface expression of the F631S mutant.

TMH6 is a "hot spot" domain for constitutively activating mutations. F631 belongs to a cluster of five consecutive residues (L629–D633) for which several constitutive activating amino acid substitutions are described (28). Intramolecular interactions between TMH5, 6, and 7 are critical for stabilizing the inactive receptor conformation (32–36). Constitutive activity by mutations is proposed to occur by breakage of hydrogen bonds between TMH6 and 7 or disturbance of hydrophobic interactions between TMH5 and 6 leading to receptor activation (35,37).

Clinical presentation of patients with the same TSHR germline mutation reveals the absence of a clear correlation between genotype and phenotype (38). Even within family members harboring the same mutation phenotypes are variable, suggesting that additional factors are required for full expression of the phenotype (39,40). Differences in thyrotoxic manifestations and development of goiter are also present in

the described family. Hyperthyroidism was diagnosed in a range of 10–19 years of age in the three children. The first two children are affected by goiter, but the youngest daughter has not yet developed goiter nor has she shown lifelong symptoms of hyperthyroidism as her siblings and mother. This new family case underlines that the same germline mutation can result in phenotypic variations regarding age of onset, severity of thyrotoxicosis and goiter, and demonstrates additional dependence of clinical manifestation of other genetic and/or environmental factors.

Premature birth and low birth weight are two other important clinical characteristics which are associated with non-autoimmune hyperthyroidism caused by activating TSHR mutations. The mean duration of gestation in patients with activating germline mutations is significantly lower than in patients with inactivating TSHR mutations causing congenital hypothyroidism (41). All three children in our investigated family were born prematurely and had low birth weight confirming this correlation (Table 1). Interestingly, a patient with congenital hyperthyroidism caused by a germline mutation, which resulted in the substitution of phenylalanine by leucine at position 631, was also born prematurely at 32 weeks of gestation with a low birth weight of 1660 g (27). These observations suggest that the TSHR might influence the timing of delivery by an yet unknown mechanism (41).

In conclusion, an autosomal dominant trait and absence of TSHR antibodies were the first clinical indications in this family. DNA analysis and subsequent functional characterization of the novel germline mutation F631S allowed us to diagnose hereditary nonautoimmune hyperthyroidism. This diagnosis has important implications for current patient

management in this family as well as for genetic counseling and presymptomatic diagnosis of hyperthyroidism in subsequent generations.

Acknowledgment

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