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Identification of a New Thyrotropin Receptor Germline Mutation (Leu^{629}Phe) in a Family with Neonatal Onset of Autosomal Dominant Nonautoimmune Hyperthyroidism*

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ABSTRACT

Constitutively activating germline mutations in the TSH receptor (TSHR) gene have been identified as a cause of autosomal dominant nonautoimmune hyperthyroidism and sporadic congenital hyperthyroidism. We report a 10-yr-old boy and his 31-yr-old mother, both presenting with a history of recurring toxic thyroid hyperplasia and no evidence for autoimmune thyroid disease. In the boy, onset of hyperthyroidism and goiter was neonatal. In the mother, onset of thyroid disease dates back to early childhood. There was no history of thyroid disease in the rest of the family. Screening for germline mutations in exon 10 of the TSHR was performed by direct sequencing of genomic DNA extracted from peripheral blood leukocytes of both patients. In the boy and his mother, an identical heterozygous TSHR mutation was identified, exchanging leucine for phenylalanine at residue 629 of the TSHR (TTG→TTT). Transient expression of the mutated TSHR construct in COS-7 cells confirmed the constitutive activity of the new TSHR germline mutation. This is the second family displaying congenital manifestation of hyperthyroidism in familial nonautoimmune hyperthyroidism. (*J Clin Endocrinol Metab* **82:** 4234–4238, 1997)

FAMILIAL clustering of hyperthyroidism is well known in Graves' disease (1). However, nonautoimmune hyperthyroidism may also segregate in families. This condition, first described by Thomas *et al.* (2), has long been a mere clinically defined entity with the diagnosis based on a family history and the finding of toxic thyroid hyperplasia in the absence of thyroid antibodies [TSH receptor (TSHR)-, thyroperoxidase-, and thyroglobulin-antibodies] and other signs of autoimmunity (endocrine opthalmopathy, pretibial myxedema, and lymphocytic infiltration in thyroid tissue). The disclosure of germline mutations in the TSHR that were first identified in two French families (3) and have since been reported in five other cases of familial nonautoimmune hyperthyroidism (5–7) has elucidated the major molecular pathomechanism of this disease. The ubiquitous presence of a constitutively activating TSHR mutation in the affected patients' thyroid gland can be expected to cause autonomous thyroid growth and function, resulting in a phenotype of hyperthyroidism and goiter (8, 9). In line with this, thyroid tissue obtained from a patient with familial nonautoimmune hyperthyroidism remained hyperfunctional after grafting in nude mice (3, 10). Moreover, progression of disease after discontinuation (2, 3, 6, 7, 11) and eventually even during antithyroid treatment (5) or relapse of thyrotoxicosis and goiter after subtotal thyroidectomy (2–5, 11) are additional clinical characteristics that may be caused by constitutively activating TSHR germline mutations. Besides the familial form of nonautoimmune hyperthyroidism TSHR germline mutations may arise *de novo* and have up to now been identified in four cases of sporadic congenital hyperthyroidism (12–15). Whereas first publications on hereditary nonautoimmune hyperthyroidism suggested that the sporadic congenital form differs from the familial form with respect to the variable and usually later onset of hyperthyroidism (ranging from 18 months to 34 yr in the same family) in the latter (3, 5), neonatal manifestation of nonautoimmune hyperthyroidism has only recently been reported in one family (7). We describe the second family with a neonatal onset of disease, thus displaying an overlap of congenital and familial nonautoimmune hyperthyroidism.

Case Report

Patient 1 (boy)

The boy was prematurely born at the 33rd week of gestation as the first child of unrelated parents. His weight was 2200 g, and his length was 43 cm. From birth on and as observed by his mother, he had increased circumference of his neck and displayed unusual irritability and easy sweating. Other clinical symptoms were frequent diarrhea and signs of advanced bone age, *e.g.* closure of the large fontanel at the age of 3 months. However, diagnosis of hyperthyroidism and goiter was not established until the age of 2 yr, when the child presented with a thyrotoxic storm. At this time, total T_3 was 7.2 ng/mL (normal, 0.08–2 ng/mL), and TSH was not measurable. A probatory discontinuation of antithyroid medication at the age of 4 yr led to the recurrence of hyperthyroidism within 8 weeks; hence, treatment with methimazole had to be reestablished and has been continued ever since. Clinically, the boy remained euthyroid, with TSH levels ranging from 0.05 to 1.1. mU/L, yet thyroid volume has increased from 15 mL (at age 8 yr) to 27 mL (at age 10 yr). A thyroid scan showed a homogeneous Tc uptake of 24%. Methimazole is now administered at a dosage of 6.25 mg/day, and L-T_4 is given at 25 μg/day. Except for a mild congenital and not progressive proptosis (16 mm in Hertel's exophthalmometry at the age of 10 yr), the

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boy has no other clinical signs or symptoms. It is noteworthy that mental and physical development in the child has been entirely normal.

Patient 2 (mother)

The mother of the boy had suggestive signs of hyperthyroidism (nervousness, low body weight despite increased appetite, and sweating) as well as goiter since early childhood. However, in her case diagnosis was also delayed. At the age of 12 yr she was first treated with methimazole. At the age of 17 yr hyperthyroidism recurred. A subtotal thyroidectomy was performed at the age of 18 yr, and L-T_4 was administered in TSH-suppressive dosage. Macroscopically the goiter was multinodular. Histological investigation of the removed thyroid tissue showed an absence of lymphocytic infiltration (Fig. 1). At the age of 21 yr while she was pregnant, she suffered a second relapse of hyperthyroidism, and antithyroid treatment with methimazole was reestablished. Despite continuation of antithyroid medication, a third relapse of hyperthyroidism as well as goiter occurred at the age of 25 yr, and radioiodine therapy was administered. Since then, the patient remained clinically euthyroid, lately taking a thyroid hormone substitution dosage of 25 μg L-T_4/day, suggesting the presence of residual functionally active thyroid tissue. As in the child, there is no clinical evidence for autoimmune thyroid disease in the mother. Repetitive screening for thyroid antibodies has been negative.

In the family history, neither the mother's parents, her three siblings, nor the child's father (Fig. 2) have been affected by thyroid disease.

Materials and Methods

DNA analysis

Genomic DNA was extracted from peripheral blood leukocytes of both patients and the boy's maternal grandparents. Two overlapping fragments encompassing the entire exon 10 of the TSHR were amplified by PCR. The primers for the N-terminal fragment (868 bp) were: forward primer, 5′-TGG CAC TGA CTC TTT TCT GT-3′; and reverse primer, 5′-GTC CAT GGG CAG GCA GAT AC-3′. The primers for the C-terminal fragment (875 bp) were: forward primer, 5′-ACT GTC TTT GCA AGC GAG TT-3′; and reverse primer, 5′-GTG TCA TGG GAT TGG AAT GC-3′ (16). PCR was performed in a 50-μL reaction mixture containing 100 ng genomic DNA, 10 mmol/L Tris-HCl (pH 8.3), 1.5 mmol/L $MgCl_2$, 50 mmol/L KCl, 0.01% gelatin, 200 μmol/L deoxy-NTP, 1 U PrimeZyme polymerase (Biometra, Gottingen, Germany), and 10 pmol of each primer. After an initial denaturation of 3 min at 95 C, samples were subjected to 30 cycles of 30 s at 95 C, 30 s at 56 C, and 1 min at 72 C, followed by a final extension step of 6 min at 72 C. PCR products were purified by polyethylene glycol precipitation (17), and double stranded sequencing of PCR products was performed with universal dye primers (Applied Biosystems, Weiterstadt, Germany) and Thermosequenase (Amersham, Braunschweig, Germany). Analysis of sequencing reactions was carried out on an automatic sequencer (Applied Biosystems 373 A).

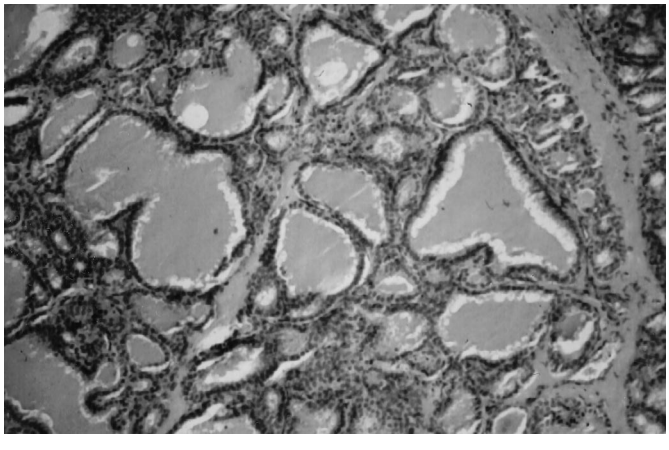

FIG. 1. Histology of the thyroid tissue obtained from patient 2 (mother) at the time of partial thyroidectomy, showing irregular nodules of varying size with partly cuboidal follicular epithelium. There is no distinct microfollicular hyperplasia. Characteristically, lymphocytic infiltrations are absent.

Cloning of the TSHR mutation Leu629Phe

Exon 10 of the TSHR gene was amplified by PCR, using genomic DNA extracted from the patient's peripheral leukocytes (described above) as template. The primers used were follows: forward primer, 5′-ATC-CTTGAGTCCTTGATGTGTAAT-3′; and reverse primer, 5′-TTA-CAAAACCGTTTGCATATACTCTT-3′. The PCR products were cloned in pUC57 (MBI Fermentas, Vilnius, Lithuania). Resulting recombinant vectors were sequenced with Thermosequenase (Amersham, Braunschweig, Germany) and dye-labeled terminators, using the primer 5′-AAGTCCGATGAGTCCAACCCG-3′, and analyzed with an automatic sequencer (Applied Biosystems 373). Constructs containing the mutant allele were cleaved with *Sca*I and *Bst*EII (positions 1439–2169). This mutated fragment was inserted into the wild-type receptor previously cloned in the expression vector pSVl. This vector with the wild-type TSHR was incompletely digested with *Sca*I (there is an additional *Sca*I site within pSVl) and subsequently with *Bst*EII. The mutated TSHR constructs were generated by replacing the *Sca*I-*Bst*EII segment in the wild-type receptor cloned in pSVl with the corresponding mutated segment amplified by PCR.

Expression of mutated TSHR constructs

For transient expression in COS-7 cells, the constructs were transfected in 100-mm dishes with 6 μg DNA of wild-type or mutated receptor constructs using the diethylaminoethyl-dextran method (18). Twenty-four hours after transfection, the cells were split and plated in six-well plates. Forty-eight hours after transfection, the cells were used for stimulation and detection of cAMP. Three 30-mm dishes were prepared for each condition.

Measurement of cAMP

Transfected cells (4×10^5/well) were washed with serum-free DMEM without antibiotics after preincubation for 30 min with the same medium containing 1 mmol/L isobutylmethylxanthine. Subsequently, the cells were incubated with or without bovine TSH (100 mU/mL; Sigma Chemical Co., St. Louis, MO) for 60 min in the presence of 1 mmol/L isobutylmethylxanthine. Thereafter, the medium was removed, and 1 mL 0,1 N HCl was added. cAMP was measured in the cell extracts with a commercial kit (Amersham, Braunschweig, Germany) according to the manufacturer's instructions. The results from a representative experiment are expressed as the mean cAMP values ± SE per 30-mm dish.

Binding assays

Transfected cells (4×10^5/well) were washed once with Hanks' solution without NaCl containing 280 mmol/L sucrose, 0.2% BSA, and 2.5% low fat milk (5). Thereafter, the cells were incubated in the same medium in the presence of 130,000 cpm [^{125}I]TSH (TRAK Assays, BRAHMS Diagnostica, Berlin, Germany; 25 μCi/μg; 40 U/mg), and the appropriate concentrations of cold TSH at room temperature for 4 h. Before the cells were solubilized with 1 N NaOH, they were washed twice with Hanks' solution. The bound radioactivity was determined in a γ-counter. All TSH or TSHR concentrations in milliunits per mL. The data were analyzed assuming a 1:1 stoichiometry for TSH binding to its receptor using the fitting module (19) of SigmaPlot 2.0 for Windows (Jandel Scientific GmbH, Erkrath, Germany).

Results

In the mother and her child an identical heterozygous TSHR point mutation was identified changing leucine for phenylalanine at codon 629 (TTG→TTT) in the sixth transmembrane segment (Fig. 2).

The expression of the mutated receptor, with the substitution of leucine for phenylalanine at position 629 in COS-7 cells, resulted in 3.5- to 4-fold higher basal values of cAMP

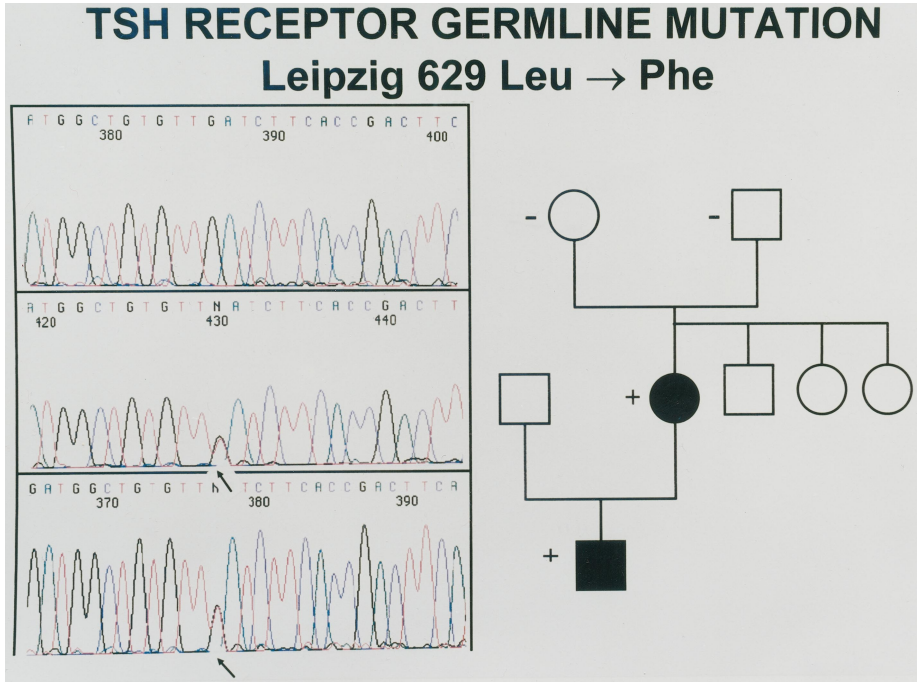

FIG. 2. Family tree and results of molecular analysis showing an identical heterozygous TSHR point mutation in patients 1 (boy) and 2 (mother) and the wild-type TSHR sequence in the maternal grandparents. The substitution of one base (TTG→TTT) results in the exchange of leucine for phenylalanine at codon 629 of the TSHR.

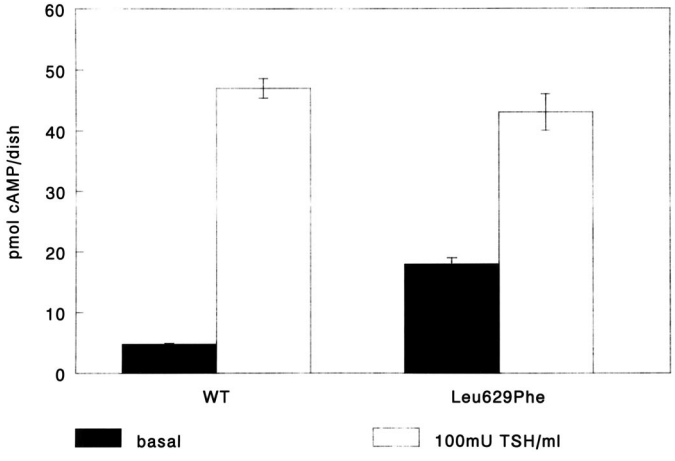

FIG. 3. Basal and stimulated (TSH, 100 mU/mL) cAMP values ± SE for the Leu^{629}Phe TSHR mutation compared to the wild-type TSHR.

accumulation (18.4 ± 1.0 pmol/well) compared to the wild-type receptor (4.8 ± 0.1 pmol/well). The Leu^{629} to Phe mutation's maximal cAMP accumulation after stimulation with 100 mU TSH/mL was similar to that of the wild-type receptor (43.6 ± 3.8 and 47.0 ± 1.6 pmol/well, respectively; Fig. 3).

Simultaneous binding experiments showed similar binding capacity values for both the wild-type and mutant receptor (0.43 ± 0.09 and 0.39 ± 0.04, respectively). The K_d values (2.57 ± 0.5 for the wild-type and 1.54 ± 0.2 for the mutant receptor, respectively) showed a slightly increased affinity of the mutant receptor for bTSH. The binding capacity values exclude overexpression of the mutant receptor as a possible reason for the increased basal cAMP accumulation.

As there was absence of thyroid disease in the rest of the family, screening for the presence of the TSHR mutation was restricted to analysis of genomic DNA extracted from the boy's maternal grandparents. As expected, only the wild-type TSHR was found in both grandparents, suggesting that the identified mutation first occurred in the mother as a *de novo* mutation and was subsequently inherited by the boy.

Discussion

The clinical findings that led us to suspect a TSHR germline mutation as the molecular etiology of hyperthyroidism in our two patients were as follows. Firstly, there were no signs or symptoms of autoimmune thyroid disease, and thyroid antibodies were negative. Secondly, symptoms were present in two generations. Thirdly, there was persistent neonatal hyperthyroidism in the child and hyperthyroidism in association with a goiter in the child and the mother. Fourthly, recurrence of thyroid disease not only occurred after discontinuation of antithyroid medication and after subtotal thyroidectomy, but progression of disease was also noted under antithyroid treatment. In agreement with other reports, these clinical signs, in fact, appear to be pathognomonic for the condition of hereditary nonautoimmune hyperthyroidism (11, 20).

Some of the TSHR mutations causing hereditary nonautoimmune hyperthyroidism have also been identified as somatic mutations in toxic thyroid nodules [Ala^{623}Val (7, 21), Leu^{629}Phe (this report and Ref. 22), Phe^{631}Leu (12, 23)], suggesting a common molecular basis of hyperthyroidism in both diseases. In addition to stimulation of thyroid function, constitutive activation of the TSHR and thereby stimulation of the cAMP cascade also promote thyroid growth (9). The associated phenotype of hyperthyroidism and goiter has been observed in our patients and is well documented for most cases of familial and sporadic congenital hyperthyroidism (2–6, 11–14) (Table 1). However, the absence of goiter was recently reported in three young patients affected by

TABLE 1. Clinical characteristics of subjects with autosomal dominant nonautoimmune hyperthyroidism

Familial (F) sporadic (S)	TSH receptor mutation	Earliest reported onset	Goiter	Surgery	Radioiodine	Relapse[a]	Author
F	$Val^{509}Ala$	8 yr	+	+	+	+	Thomas *et al.* (2) Duprez *et al.* (3)
F	$Cys^{672}Tyr$	18 months	+	+	+	+	Leclere *et al.* (11)
F	$Ser^{505}Arg$	Childhood	+	+	+	+	Horton *et al.* (4)
F	$Asn^{670}Ser$	17 yr	+	?	+	+	Tonacchera *et al.* (5)
F	$Asn^{650}Tyr$	14 yr	+	+	?	+	
F	$Arg^{528}His$	2 months	?	+	−	+	Köhler *et al.* (6)
F	$Leu^{629}Phe$	Neonatal	+	+	+	+	Führer *et al.* (this report)
F	$Ala^{623}Val$	Neonatal	+	+	−	+	Schwab *et al.* (7)[b]
S	$Phe^{631}Leu$	Neonatal	+	+	+	+	Kopp *et al.* (12)
S	$Met^{453}Thr$	Neonatal	+	−	−	−	De Roux *et al.* (13)[c]
S	$Ser^{505}Asn$	Neonatal	+	+	−	+	Holzapfel *et al.* (14)
S	$Val^{597}Leu$	Neonatal	−	+	−	−	Esapa *et al.* (15)[d]

?, Not described.

[a] Relapse of hyperthyroidism after or during antithyroid treatment or surgery (partial thyroidectomy) in one or more family members with a TSHR germline mutation.

[b] Goiter not present in the neonatal period.

[c] Period of follow-up not described.

[d] Total thyroidectomy at 14 months.

hereditary nonautoimmune hyperthyroidism (7, 15). In one patient, hyperthyroidism occurred during the first year of life, and as it was resistant to antithyroid treatment, a total thyroidectomy was performed at the age of 14 months (15). In the two other patients, hyperthyroidism was of neonatal onset and successfully treated with antithyroid medication over a period of currently 4 yr in the eldest child (7). It is noteworthy that the patient's mother also carries the TSHR germline mutation ($Ala^{623}Val$) and herself has suffered from relapses of both hyperthyroidism and goiter.

These observations could suggest that constitutive activation of the TSHR might first manifest as hyperthyroidism and only secondly as hyperthyroidism and goiter in some cases. This would imply involvement of additional regulating factors or a longer latency for the manifestation of growth stimulation than stimulation of thyroid function. Moreover, different effects of activating TSHR mutations on the phospholipase C-dependent cascade have been reported (20, 23). As control of cell proliferation in vertebrates is also linked to this pathway (8), activation of the inositol phosphate cascade by some TSHR mutations could explain differences in growth stimulation.

Another intriguing issue is the age at onset of familial nonautoimmune hyperthyroidism. A considerable variation has been documented for all eight families, each with a different TSHR germline mutation and, more importantly, it was also observed among family members with the same TSHR mutation (2–7, 11) (Table 1). There has been some speculation that the level of constitutive activity of a TSHR mutation may influence the phenotype of disease (5). However, parallel transfection of 11 TSHR mutations (20) showed no correlation between *in vitro* findings and reported *in vivo* data, *e.g.* the onset of disease: higher specific constitutive activity ($Cys^{672}Tyr > Val^{509}Ala = Phe^{631}Leu$) was not con-sistently associated with earlier onset of hyperthyroidism [18 months ($Cys^{672}Tyr$), 8 yr ($Val^{509}Ala$), neonatal ($Phe^{631}Leu$)]. Likewise, lower specific constitutive activity ($Ala^{623}Val < Ser^{505}Arg$) did not correlate with later onset of hyperthyroidism [neonatal ($Ala^{623}Val$), $Ser^{505}Arg$ (childhood)] (3–5, 7, 11). Which other factors may then determine the clinical manifestation of a constitutively activating TSHR germline mutation? Difference in expressivity of mutations in dominant diseases is commonly observed and may be attributed to genetic and epigenetic factors as well as environmental influences (25). However, as members of the same family that can be expected to experience a similar environmental background (in particular with respect to iodine supply) still display a variable onset of hyperthyroidism (2–7), it appears likely that determining factors are, rather, of an endogenous nature.

It is noteworthy that hyperthyroidism in one of our patients (the mother) recurred during pregnancy. A stimulatory effect of hCG on the TSHR is well documented *in vitro* (26, 27) and is presumably related to clinical conditions such as hyperemesis gravidarum (28) as well as hyperthyroidism in choriocarcinoma (29) and hCG-producing germ cell tumors (30). Therefore, an influence of hCG on the relapse of thyroid disease during pregnancy cannot be excluded in the mother.

Several clinical implications evolve from the identification of a constitutively activating TSHR germline mutation in our patients. The first concerns redefinition of an adequate treatment of the boy's thyroid disease. Although the child has remained euthyroid with antithyroid medication, progressive growth of goiter is now a prominent problem. As previous reports, in particular the follow-up of the first described family with nonautoimmune hyperthyroidism (2, 3, 11), have shown, a near-total thyroidectomy rather than a partial thyroidectomy, leaving thyroid tissue with the constitutively activating TSHR germline mutation, is the treatment of choice to prevent relapses. Antithyroid medication cannot be recommended on a longer term (or life-long) basis because hyperthyroidism relapsed under antithyroid treatment in several patients (4, 5, 12, and 14 and this report).

Although the boy's mother has now remained euthyroid for 4 yr, the relatively low thyroid hormone substitution dose (25 μg/day) indicates the presence of substantial residual

thyroid tissue. Therefore, this patient requires follow-up. Eventually further ablative thyroid treatment may be necessary.

The finding of a constitutively activating TSHR mutation also concerns the implication for genetic counselling. As the mutation is autosomal dominantly inherited, there is a 50% risk of transmitting the affected gene to the offspring. In this context, a molecular analysis of genomic DNA extracted from a routinely obtained blood sample will allow preclinical diagnosis.

In summary, we describe a family with hereditary nonautoimmune hyperthyroidism of congenital onset due to a new TSHR germline mutation. The increasing number of case reports on both familial and sporadic congenital nonautoimmune hyperthyroidism indicates that these disorders may be more frequent than hitherto thought. In view of the different therapeutic approach, and as it is inherited, a TSHR germline mutation should be suspected in all patients with a relapsing or treatment-resistant course of nonautoimmune hyperthyroidism. Moreover, differentiation between sporadic congenital and familial nonautoimmune hyperthyroidism may no longer be justified because of the common molecular etiopathogenesis and because the familial form may manifest as severe congenital hyperthyroidism (Ref. 7 and this report). Therefore, these hereditary thyroid disorders should be classified as autosomal dominant nonautoimmune hyperthyroidism.

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