Severe Congenital Hyperthyroidism Caused by a Germ-Line *neo* Mutation in the Extracellular Portion of the Thyrotropin Receptor

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ABSTRACT
Giant of function mutations in the TSH receptor (TSHR) have been identified as the molecular basis for congenital and acquired forms of autonomous thyroid function. Herein, we report the molecular characterization of a case of severe congenital hyperthyroidism with a history of hyperthyroidism in the paternal aunt and the paternal grandmother, who were both found to be heterozygous for a mutation (R528H) located in exon 10 of the TSHR gene. Functional expression of the mutant TSHR-R528H in COS-7 cells, however, did not result in constitutive activity of the TSHR. Subsequent analysis of exons 1–9 led to the detection of an additional heterozygous mutation (S281N) in the patient, but not in other family members. Interestingly, the latter mutation is located in the extracellular domain of the TSHR, and functional studies revealed a marked increase in basal cAMP levels when the mutant receptor was expressed in COS-7 cells. To address the question of whether both mutations were present on the same allele, a double mutant TSHR (S281N/R528H) was generated and characterized. These functional studies in conjunction with RT-PCR analysis of thyroid tissue obtained from subtotal thyroidectomy performed at the age of 6 yr revealed that the patient bears two distinct mutations on different alleles: the familial paternal R528H mutation to be regarded as a polymorphism and a de novo mutation (S281N) on the maternal allele accounting for the clinical picture. Thus, the main conclusions to be drawn from this case are 1) a search for mutations in cases of congenital nonautoimmune hyperthyroidism should not remain restricted to exon 10 of the TSHR gene, because germ-line gain of function mutations of the TSH receptor can be located outside of the transmembrane core of the receptor; and 2) this case illustrates the necessity for careful functional characterization of any novel mutation before a causal relationship to hyperthyroidism can be established. (J Clin Endocrinol Metab 83: 1431–1436, 1998)

CONGENITAL hyperthyroidism is a rare disease that is usually caused by transplacental passage of maternal TSH receptor (TSHR)-stimulating autoantibodies in Graves’ disease (1). Sporadic cases of congenital nonautoimmune hyperthyroidism with onset of disease during childhood or even later (5, 6) were found to be caused by mutations within the TSHR gene leading to constitutive activity of the receptor. To date, all germ-line mutations responsible for acquired and congenital nonautoimmune hyperthyroidism were localized in exon 10 encoding the entire transmembrane region of the TSHR (Fig. 1). Only very recently has a somatic mutation in a hyperfunctioning thyroid adenoma been identified by an amino acid exchange in the extracellular portion of the TSHR (7).

In the present study, we examined a girl with nonautoimmune congenital hyperthyroidism and a history of hyperthyroidism of adult onset in the paternal aunt and maternal grandmother. Interestingly, all previously reported cases of congenital nonautoimmune hyperthyroidism had occurred sporadically, probably because the severity of hyperthyroidism had prevented familial transmission. These considerations notwithstanding, we initially hypothesized that congenital hyperthyroidism was caused by a germ-line mutation of the TSHR gene leading to constitutive receptor activity in several family members, and we set out to characterize the molecular TSHR defect in our patient.

Case Report
The female patient was born in the 36th week of gestation with a birth weight of 2520 g (50th percentile) and a length of 49 cm (90th percentile). Both parents were healthy and negative for a history of thyroid disease. However, in three generations, several members of the paternal family developed thyroid disease. In particular, hyperthyroidism had been
reported for the paternal grandmother and paternal aunt (Fig. 2). The paternal grandmother and paternal aunt had always been negative for TSHR antibodies. Both had low titer antithyroperoxidase antibodies (300 and 1000 U/mL, respectively).

Retrospectively, the parents reported that restlessness, frequent crying, sweating, and diarrhea had been present in the first weeks of life. The persistence of these symptoms led to pediatric consultation after 4 weeks, but the diagnosis of hyperthyroidism was delayed until the age of 4 months. She presented with severe irritability, sweating, diarrhea, tachycardia (180–200/min), an elevated blood pressure amplitude (105/53 mm Hg), and staring eyes without further signs of ophthalmopathy, yet no signs of thyroid enlargement. Free thyroid hormone values were profoundly elevated [free T4 (fT4), 7.5 ng/dL (96.8 pmol/L); free T3 (fT3), 26.6 pg/mL (40.9 pmol/L)]. TSH was suppressed to 0.04 mU/L and showed no increase after TRH administration. Retrospective analysis of the fT4 level in a filter paper specimen obtained on the fifth day of life revealed a concentration of 3.9 ng/dL (50.7 pmol/L), whereas the TSH level had previously been reported as normal (5 mU/L) by the neonatal screening laboratory. Tests for antithyroid antibodies were negative in the patient as well as in both parents, who had normal thyroid hormone levels (Table 1).

Although symptoms promptly resolved with thiamazole and propranolol treatment, they reoccurred whenever an attempt was made to reduce the dose of antithyroid drug therapy. After 1 yr, a neurosurgical intervention became necessary because of craniosynostosis. The failure to prevent this complication despite antithyroid drug treatment in conjunction with the observation of immediate relapses of hyperthyroidism after dose reduction led to the decision to perform thyroidectomy at the age of 6 yr. Thereafter, thyroid hormone values remained in the normal range without antithyroid drug treatment, but TSH levels were suppressed.

**Methods**

Neonatal TSH, serum TSH, serum T4, and serum T3 were measured with an immunofluorometric assay (Delfia, Wallac, Freiburg, Germany) or RIA. Serum fT4, fT3, antithyroperoxidase, antithyroglobulin, and TSHR antibodies were measured by RIA (Dynotest, Brahms Diagnostica, Berlin, Germany).

For mutation analyses, genomic DNA, PCR, single strand conformation polymorphism (SSCP), and sequencing were performed as described previously (8).

**Construction of mutant TSHR genes**

To characterize functional properties of the novel mutant TSHRs (Fig. 1) detected in this study, mutations were created by standard PCR mutagenesis techniques (9) using the human TSHR expression plasmid, TSHR-pcD-PS (8), as a template in which the Bsu36I/BstEII segment had been replaced by a corresponding fragment amplified from genomic DNA. PCR fragments containing the S281N and R528H mutations were digested and used to replace the corresponding KpnI/MscI and MscI/Bsu36I fragments, respectively. The identities of the different constructs and the correctness of all PCR-derived sequences were confirmed by restriction analysis and dideoxy sequencing.

**Cell culture, expression, and functional characterization of TSHR constructs**

COS-7 cells were grown and transiently transfected as described previously (8).

For inositol phosphate (IP) and cAMP measurements, cells were split into 12-well plates (2 × 10⁵ cells/well) and transfected on the subsequent day. Approximately 24 h after transfection, 2 μCi/mL myo-[³H]inositol (18.6 Ci/mmol; Amersham, Aylesbury, UK) or
[\textsuperscript{3}H]adenine (28.8 Ci/mmol; DuPont-New England Nuclear, Brussels, Belgium) were added to the growth medium. After a 20- to 24-h labeling period, IP and cAMP accumulation assays were performed as described previously (10, 11).

For radioligand binding studies, cells were harvested 72 h after transfection, and displacement binding assays were performed with membranes prepared as previously described (12). Incubations were carried out in a volume of 0.25 mL for 1 h in the presence of 40,000 cpm \([\textsuperscript{125}I]\)bovine (b) TSH (54 \text{ mCi}/\text{mg}; Brahms Diagnostica) and bTSH ranging from 0.001–100 mU/mL. Nonspecific binding was defined as binding in the presence of 100 mU/mL bTSH. Membranes and bound ligand were separated from unbound ligand by centrifugation (10,000 \text{ g} for 10 min) through a silicon oil layer according to the procedure of McArdle et al. (13). The protein content of samples was determined by the method of Bradford (14). Binding data were analyzed by a nonlinear least squares curve-fitting procedure using the program Ligand (15).

**Ribonucleic acid (RNA) preparation and RT-PCR**

Tissue obtained from subtotal thyroidectomy was subjected to RT-PCR. About 250 mg thyroid tissue were homogenized with a mortar and pestle under liquid nitrogen. RNA was prepared using the Trizol method (Life Technologies, Eggenstein, Germany). First strand complementary DNA was synthesized by RT (Stratagene, Heidelberg, Germany), using an oligo(dexoxythymidine) primer as recommended. Then, a DNA fragment spanning the entire nucleotide sequence of exons 9 and 10 was amplified.
by PCR with the sense primer 5'-GAC GTG TCT CAA ACC AG-3' and
the antisense primer 5'-GCA GAC GAT GAC GAA GG-3'. The
purified PCR product was cloned into the pCR-TOPO vector (Invitrogen,
Leek, The Netherlands), and 10 clones were sequenced.

**Results**

**Identification of TSHR mutations**

Five DNA fragments encompassing the entire exon 10 of
the TSHR of the patient were amplified by PCR. As indicated
in Fig. 3B, the PCR products representing a 401-bp segment
(nucleotide position 1397–1797) showed marked differences
in electrophoretic mobility compared with amplification
products from her healthy brother or mother (see Fig. 3B).
Further investigation of all family members available identi-
fied the sister, father, paternal aunt, and paternal grand-
mother as heterozygote carriers of the abnormality in exon
10. Direct sequencing of the segment was performed, and a

G to A transition in codon 528 was identified, leading to an
R to H exchange. The mutation in exon 10 within one allele
led to the loss of a HhaI restriction site in the 401-bp fragment
that could now be used to identify this mutation in other
family members (see Fig. 3C).

**Expression and functional characterization of the wild-type
and mutant TSHR**

To functionally characterize this novel TSHR mutation, we
expressed wild-type and mutant TSHRs in COS-7 cells and
performed cAMP and IP accumulation assays, as well as [125I]TSH
binding assays in parallel. As shown in Fig. 4A, COS-7 cells
transfected with the R528H mutant displayed lower basal
cAMP levels compared with the wild-type TSHR. Maximum
increases in cAMP levels after bTSH stimulation (100 mU/mL)
were reduced by about 40% in cells expressing TSHR-
R528H (Fig. 4A and Table 2). As functional expression of
TSHR R528H could not offer an explanation for the occur-
rence of severe congenital hyperthyroidism in our patient,
we extended sequence and SSCP analyses to exons 1–9 of all
family members. As shown in Fig. 3D, a heterozygous, ab-
normally migrating fragment was found in the patient’s exon
9, but not in other family members. Sequence analysis re-
vealed a G to A transition in codon 281, resulting in an S to
HYPERTHYROIDISM CAUSED BY neo MUTATION IN TSH RECEPTOR

In the present case of congenital hyperthyroidism, the grandmother’s hyperthyroidism leading to thyroidectomy in adulthood and the aunt’s hyperthyroidism requiring antithyroid drug treatment since adolescence first suggested inheritance with variable onset of disease. The R528H mutation that was identified in these individuals showed no increased constitutive receptor activation in expression experiments. An alignment of the TSHR sequence of the second intracellular loop with corresponding amino acid sequences of other glycoprotein hormone receptors showed that R528 in the TSHR is naturally replaced by the amino acid H at the corresponding position of the receptor for LH. Therefore, the R528H mutation could not account for congenital hyperthyroidism in our patient.

It has been demonstrated that a deletion mutation in the extracellular portion of the TSHR results in constitutive receptor activity (16), and in a recent study, a mutation in exon 9 (S281N) has been found in a thyrotoxic nodule. The identical de novo mutation (S281N) was found in our patient and constitutively activated the Gs/adenyl cyclase. Thus, we describe the first case of congenital nonautoimmune hyperthyroidism caused by a de novo germ-line mutation (S281N) in the extracellular domain of the TSHR.

The current concept of TSHR activation has to be extended to a possible involvement of the receptor’s ectodomain. As the TSH receptor displays significant constitutive activity even in the unliganded state, it is possible that a negative constraint exerted by the extracellular unliganded domain usually maintains receptor quiescence. A mutation within the extracellular domain disrupting a crucial three-dimensional structure necessary for receptor silencing may then lead to constitutive activity.

In our patient, the decision for thyroidectomy was based on clinical grounds before mutational analysis and in vitro characterization. An early time point for thyroidectomy in patients with persisting nonautoimmune hyperthyroidism

### Table 2. Functional characterization of wild-type (wt) and mutant TSHRs

<table>
<thead>
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<td></td>
<td>Basal (cpm)</td>
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<td>pcD-PS</td>
<td>208 ± 22</td>
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N exchange. Sequencing of the other TSHR exons revealed no additional mutations in the TSHR gene of the investigated family members.

Since this study was performed, Duprez et al. (7) identified a constitutively activating mutation in the same codon of exon 9 in an autonomous adenoma. In accordance with this observation, Fig. 4A shows that the basal cAMP levels of COS-7 cells transfected with the S281N mutant were significantly elevated. As the TSHR is known to activate the cAMP and phosphoinositide signaling cascades, all TSHR constructs were analyzed in an IP accumulation assay. We demonstrated that the mutant TSHRs were not constitutively active in terms of IP formation. The S281N and R528H mutations did not alter maximal IP levels after stimulation with TSH (see Fig. 4B and Table 2).

For characterization of ligand binding properties, wild-type and mutant TSHRs were expressed in COS-7 cells, and [125I]TSH displacement studies were performed. No significant differences between the K\textsubscript{d} values were observed among the three mutants compared with the wild-type TSHR (Table 2). Membrane expression of all mutant TSHRs was reduced to 35–54% of the wild-type receptor expression level (binding capacity, 315 fmol/mg membrane protein; n = 4; Table 2).

Furthermore, we constructed a recombinant TSHR containing both mutations. Expression studies revealed similar functional properties of the R528H/S281N mutant and the wild-type receptor (Table 2). To unequivocally clarify the allelic localization of both mutations in our patient, we extracted RNA from thyroid tissue that had been collected at the time of thyroidectomy and performed RT-PCR studies.

Therefore, PCR fragments spanning the entire exons 9 and 10 were subcloned, and 10 individual clones were sequenced. As previously indicated by functional studies, the patient was found to be a compound heterozygote, i.e. individual clones contained either the R528H or the S281N mutation. Thus, a de novo germ-line mutation (S281N) on the maternal side was identified as the cause of severe congenital hyperthyroidism in this patient, whereas the parental R528H mutation identified in several family members affected by thyroid disorders had to be regarded as a polymorphism.

**Discussion**

In published cases of congenital hyperthyroidism the disease was caused by de novo mutations (M453T, F631L, S505D) only present in the affected infants (2–4) and not in the other family members. The first descriptions of pedigrees with autosomal dominant traits due to germ-line mutations (S505R, V509A, N650Y, N670S, C672Y) of the TSHR were presented by Vassart and co-workers (5, 6). A clear correlation between genotype and phenotype was difficult to establish because individuals of the same family harboring identical mutations became overtly hyperthyroid either during infancy or later in adulthood (5), but none of the affected individuals presented with congenital hyperthyroidism. One may speculate that the severity of hyperthyroidism in the congenital cases up until now has prevented familial transmission, because there is no overlap between the mutations leading to either severe congenital forms hyperthyroidism or toxic adenomas and the mutations found in familial nonautoimmune hyperthyroidism with an autosomal dominant trait of inheritance.

In the present case of congenital hyperthyroidism, the grandmother’s hyperthyroidism leading to thyroidectomy in adulthood and the aunt’s hyperthyroidism requiring antithyroid drug treatment since adolescence first suggested inheritance with variable onset of disease. The R528H mutation that was identified in these individuals showed no increased constitutive receptor activation in expression experiments. An alignment of the TSHR sequence of the second intracellular loop with corresponding amino acid sequences of other glycoprotein hormone receptors showed that R528 in the TSHR is naturally replaced by the amino acid H at the corresponding position of the receptor for LH. Therefore, the R528H mutation could not account for congenital hyperthyroidism in our patient.

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regardless of the results of sequencing and in vitro studies appears to be mandatory because three of the published cases remained hyperthyroid despite prolonged antithyroid drug treatment (3, 4) and suffered from irreversible sequelae. Analysis of the TSHR gene may help to justify the decision of thyroidectomy, but it has to be taken into account that the results of in vitro findings cannot be directly transferred to the in vivo situation, which is modulated by genetic and epigenetic factors. However, in any case of novel TSHR mutations, thorough functional characterization has to prove the causal relationship to the clinical picture. In this case, the detection and functional analysis of the additional mutation in exon 9 of the TSHR allowed us to conclude that there is no increased risk of developing hyperthyroidism for the patient’s father and sister.

Acknowledgments

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References