CLINICAL STUDY

A novel germline mutation in the TSH receptor gene causes non-autoimmune autosomal dominant hyperthyroidism

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Abstract

Objective: Clinical and genetic investigations were undertaken in a case of familial hyperthyroidism, with onset of thyrotoxic symptoms varying between childhood/adolescence.

Methods: Automatic sequence analysis was carried out of the TSH receptor (TSHR) gene. Functional studies were undertaken of mutant TSHR in transient expression experiments in COS-7 cells including the evaluation of cAMP accumulation and of protein expression by flow cytometry, as well as the calculation of specific constitutive activity (SCA).

Results: In four affected cases, the age of onset of thyrotoxic manifestations of non-autoimmune origin varied between 5 and 18 years. The disease transmission was typically autosomal dominant. TSHR gene sequence revealed the presence of a germline heterozygous substitution at codon 597 leading to the novel mutation V597F. This residue is located in the 5th transmembrane domain of the receptor protein in a critical region for membrane targeting and signal transduction. Functional studies of the V597F mutant indicate an 11-fold increase in SCA, associated with a reduction in receptor protein expression on the cytoplasmic membrane.

Conclusions: Description was made of a family with non-autoimmune autosomal dominant hyperthyroidism carrying a novel mutation of TSHR leading to the increment in specific constitutive activity. Factors that may influence the clinical expression of TSHR germline mutations are discussed.

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Introduction

Autoimmunity (Graves' disease) is the major cause of thyrotoxicosis in early infancy and childhood (1). Germline gain-of-function mutations of thyrotropin (TSH) receptor (TSHR) have been found to cause non-autoimmune hyperthyroidism with neonatal to juvenile onset (2). The sporadic cases so far reported presented already at birth or in infancy with severe thyrotoxic manifestations and goiter (3–9), whereas a delayed and progressive onset of hyperthyroidism with milder clinical manifestations was generally observed in the cases with inherited mutation of the TSHR gene (10–13). The reasons for such a variable age of onset and degree of disease manifestation may be attributed either to the type of the mutation or to other endogenous or exogenous factors. Typically, the disease is persistent with immediate relapses after withdrawal of anti-thyroid drugs, and sporadic cases are frequently resistant to medical treatment. Therefore, total thyroidectomy is the treatment of choice in these situations.

We report a new family with non-autoimmune autosomal dominant hyperthyroidism due to a novel germline mutation of the thyrotropin receptor gene.

Materials and methods

Case report

The proband is a female born after normal gestation. Her family originates from the Romagna region in Italy. She was seen at 5 years of age for high stature accompanied in the last months by progressive worsening of symptoms such as hyperactivity, irritability, and palpitations. Physical examination showed mild signs of thyrotoxicosis with a small goiter: her heart rate was 120 beats/min, blood pressure was 130/70 mmHg. Her body weight was 34 kg, and her height was above the 97th centile: 129 cm. She had no sign of sexual maturation and no skin lesions. The biochemical investigations showed TSH: <0.01 mU/l (normal value (n.v.) 0.24–4.0); free thyroxine (FT₄):

(16). Cells were then grown in DMEM/10% FCS for 48 h before cAMP determination and flow cytometry experiment.

cAMP determination

The culture medium was removed and replaced with Krebs-Ringer-HEPES (KRH) buffer, 0.5% BSA, for 30 min at 37 °C. Then cells were incubated at 37 °C for 60 min in fresh KRH buffer, 0.5% BSA, supplemented with 0.5 mmol/l isobutylmethylxanthine (IBMX) and the indicated concentrations of bovine (b) TSH (Sigma-Aldrich srl, Milan, Italy). The medium was removed and the reaction stopped by adding 0.1 mol/l HCl. Samples were dried and cAMP was measured using a commercial assay kit (RIANEN, NEN Life Science Products Inc., Boston, MA, USA). The results were expressed in pmoles/dish.

Flow cytometry

Flow cytometry experiments were performed as previously described (17). For permeabilization, cells were incubated with PBS-paraformaldehyde 2% in ice for 10 min, washed once with PBS-BSA 0.1% and treated with 2% saponine at room temperature for 30 min. Permeabilized and non-permeabilized cells were labeled with 3G4 monoclonal antibody recognizing a linear epitope, and with BA8 antibody recognizing a conformational epitope, both within the ligand-binding domain of TSHR (18).

Computation of specific constitutive activity

The fold cAMP constitutive activity above mock-transfected COS-7 cells was calculated for each receptor construct, and then normalized for their own level of expression, as previously described (19). Specific constitutive activity (SCA) of the mutant was then expressed as fold-increase over the wild-type (19).

Statistical analysis

Data were analyzed by Student's *t*-test and analysis of variance (ANOVA), as appropriate. *P* values <0.05 were considered statistically significant. Results are expressed as means ± standard deviation (s.d.).

Results

DNA analysis

Automatic sequencing of DNA obtained from peripheral whole blood of the proband revealed a heterozygous G to T transversion at codon 597 in the TSHR gene leading to the missense substitution V597F (Fig. 2). This codon is located in the fifth transmembrane domain of the receptor. Both the father and the half-

Figure 2 Electropherogram of dye terminator sequencing around codon 597 (arrow) of the TSHR gene in the proband and in the other affected members of the family. The double peak indicates the presence of two bases (G or T) at that position.

sister of the proband were heterozygote for the same substitution, whereas the TSHR sequence was of the wild-type (WT) in the mother and the paternal grandmother (Fig. 1).

Functional studies of mutant receptor

Flow cytometry experiments, one of which is shown in Fig. 3, indicate that the synthesis of TSHR-WT and TSHR-V597F constructs are not significantly different as demonstrated in permeabilized cells, but the receptor carrying the activating mutation displays a reduced expression level on the cell surface. The entity of this reduction is 60% of that observed with normal receptor using the 3G4 antibody (Fig. 3A). Expression of the V597F mutant was even lower (25% vs wild-type) when the BA8 monoclonal antibody directed against a conformational epitope was used (Fig. 3B).

In order to evaluate the activity of the V597F mutant receptor, we measured basal and TSH-stimulated cAMP accumulation in transiently transfected COS-7 cells in several experiments (*n* = 7). As shown in Fig. 4, expression of the V597F mutant receptor always resulted in a marked increase in basal cAMP production when compared with the wild-type receptor or mock-transfected cells. The V597F mutant maintained the ability to respond to TSH stimulation, although the increment in cAMP production at 10 U/l bTSH is 2.4-fold lower than that of wild-type (Fig. 4). Maximal cAMP stimulation was reached at 10 U/l bTSH in both cases. On this basis, calculation of SCA (19) showed an 11.4 ± 0.4 - or a 4.6 ± 0.2 -fold increase over the wild-type receptor in intact and permeabilized cells respectively.

Discussion

We report a new case of non-autoimmune autosomal dominant hyperthyroidism. In this family, thyrotoxic manifestations and goiter had a childhood to juvenile onset, ranging from 5–18 years of age in the four

Figure 3 Representative flow cytometry experiment, using 3G4 (A) and BA8 (B) monoclonal antibodies (Ab), for the analysis of wild-type (wt) and V597F TSHR protein expression in intact and permeabilized cells. Results with mock transfected cells (pTarget) are also shown. These data demonstrate a reduced expression of V597F mutant TSHR protein on the membrane of intact COS-7 cells.

Figure 4 Functional studies of wild-type (wt) TSHR and V597F mutant in transient transfection in COS-7 cells. The results of basal and TSH-stimulated cAMP accumulation in one representative experiment are shown (means $\pm$ s.d. of three different determinations). pTarget, mock transfected cells. * $P < 0.05$ vs pTarget; ** $P < 0.05$ vs wild-type TSHR.

affected members. Automatic sequencing of the TSHR gene using genomic DNA from the proband and the other affected relatives revealed the presence of a novel heterozygous germline transversion at codon 597 (GTC to TTC). This mutation leads to the amino acid substitution V597F in the fifth transmembrane domain of the receptor. Molecular and functional studies allowed us to reach the correct diagnosis of non-autoimmune autosomal dominant hyperthyroidism.

The biological function of this mutant receptor was evaluated by transient transfection in COS-7 cells, in comparison with normal receptor. The V597F substitution causes a marked increase in ligand-independent receptor activity, despite the fact that flow cytometry experiments showed a marked reduction in cell surface expression of this mutant in COS-7 cells compared with the wild-type receptor. The reduction in cell surface expression could be due to an abnormal folding of the mutant receptors preventing the correct membrane targeting. The lower expression levels given at the analysis with the monoclonal antibody against a conformational epitope, such as BA8 (18), may also be due to the altered immunoreactivity of the mutant receptor. The presence of the V597F mutant on the plasmatic membrane is confirmed by the conserved response to TSH stimulation. The same codon was recently found mutated with a different substitution (V597L) in one infant with severe thyrotoxicosis (8). Functional experiments of the V597L TSHR mutant showed a dramatic increase in ligand-independent

receptor activity associated with the severe impairment of membrane targeting, leading to a diminished response to TSH stimulation. The increase in specific constitutive activity of the V597L mutant was markedly higher than that observed in the case of V597F (28.9- vs 11.4-fold over wild-type in intact cells). As in other G protein-coupled receptors (20), the intramolecular interactions between hydrophobic residues in the fifth and sixth transmembrane domains would contribute to the structural inhibitory constraint keeping the TSHR in its basal state (21). V597 residue is one of the hydrophobic residues involved in this structural inhibitory constraint and mutations at this residue are expected to alter constitutive receptor activity. However, the differences in specific constitutive activity between V597L and V597F are difficult to explain. In fact, both leucine and phenylalanine have non-polar side chains like valine, and the bulkier side chain of phenylalanine would be expected to produce a more profound disruption of the structural inhibitory constraint.

Although caution is advised in interpreting data obtained in a heterologous system, the reported differences in the *in vitro* functional characteristics of the two mutants affecting the same V597 residue are mirrored by the clinical phenotype recorded in the affected patients. In fact, the V597L mutation was found in one sporadic case with infantile onset (at 9 months of age) of severe thyrotoxic features (8), whereas V597F is associated with a childhood to juvenile onset of thyrotoxic features. Germline mutations causing dramatic increases in TSHR constitutive activity causing severe thyrotoxicosis since early infancy (or even congenital) (3–9) may be associated with intellectual impairment and craniosynostosis in later years (22), and familial vertical transmission may become unlikely. Alternatively, this would not be prevented in the case of germline mutations with less dramatic effects on TSHR function (10–13). Consistently, the inherited TSHR mutations generally present later in life (typical childhood or juvenile onset) with mild to moderate thyrotoxic manifestations. Hyperstaturism was described in our proband and bone was severely affected, indicating that, despite mild and unrecognized clinical symptoms, tissue thyrotoxicosis had been long-standing. Increased growth velocity was, at least in part, mediated by the thyroid hormone-dependent induction of IGF-I secretion from the liver, perhaps through the stimulation of hepatic growth hormone receptor gene expression, as already seen in the chicken (23). Consistently, increased IGF-I secretion reverted to normal during MMI treatment.

Increased medical awareness and improvement in diagnostic testing (such as availability of ultrasensitive TSH assay) (24) throughout the last decades could have allowed the earlier diagnosis in the proband. Nevertheless, the clinical expression of the same alteration of the TSHR gene in the different members of one family may also be influenced by several

environmental (such as modifications in iodine intake) and/or endogenous (such as sex) factors. During the 1980s, when the father and aunt of our proband both underwent thyroidectomy and before the prophylaxis with iodized salt was well-established, the mean urinary excretion of iodine was as low as 58.1 µg/l in the Romagna region (25). Accordingly, the diagnosis of thyrotoxicosis was reached at 15 and 18 years in the older members of the family and at 5 years in the proband. Differently from what is usually seen in sporadic cases, hyperthyroidism could be controlled by high doses of methimazole. However, progressive thyroid growth is expected through the years and strict ultrasound monitoring is advocated during treatment in order to program thyroid surgery before the formation of a large goiter, as already seen in her affected relatives.

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