A Novel Thyrotropin Receptor Germline Mutation (Asp617Tyr) Causing Hereditary Hyperthyroidism

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Abstract. Constitutively activating germline mutations of the thyrotropin receptor (TSHR) gene have been identified as a molecular cause of hereditary nonautoimmune hyperthyroidism. We describe here a Japanese kindred with two affected individuals who showed overt hyperthyroidism and mild goiter in the absence of TSHR antibodies. A novel heterozygous germline point mutation, identified in both individuals, resulted in an amino acid substitution of aspartic acid for tyrosine at codon 617 (Asp617Tyr) in the third intracellular loop of the TSHR. Screening of 7 additional family members led to the identification of the same mutation in 4 relatives: 1 had undergone thyroidectomy due to hyperthyroidism but 3 were asymptomatic with subclinical hyperthyroidism.

In vitro functional studies of the Asp617Tyr TSHR demonstrated a constitutive activation of the cyclic adenosine monophosphate pathway, but not of the inositol phosphate cascade, with data similar to those of Asp619Gly, the first constitutively activating mutant TSHR identified. Treatment with inorganic iodine for 7 months successfully relieved all symptoms of hyperthyroidism in both patients.

Key words: Thyrotropin receptor, Germline mutation, Nonautoimmune hyperthyroidism, Adenylyl cyclase activation, Third intracellular loop

IN iodine sufficient regions, including Japan, the most common cause of hyperthyroidism in adult is Graves’ disease, in which autoantibodies against the thyrotropin receptor (TSHR) overstimulate the thyroid gland. The pathogenesis of nonautoimmune hyperthyroidism had been obscure until constitutively activating germline mutations in the TSHR were identified as a cause of hereditary hyperthyroidism in two families [1] and in one sporadic case [2]. Subsequently, 29 germline TSHR mutations in 19 families [1, 3–18] and 10 individuals with sporadic occurrence [19, 20] were reported. Clinical characteristics in familial cases include: autosomal dominant transmission, hyperthyroidism with a variable age of onset, hyperplastic goiter of variable size, and absence of clinical or biological features of autoimmunity [1, 3–18]. Even among family members harboring the same mutation, clinical features are variable, suggesting that environmental factors including iodine intake, or other genetic factors may be required for ful expression of the phenotype. Despite these differences in expression, ablative therapy (surgery or radiiodine) is commonly required to achieve long-term remission.

TSHR belongs to the large superfamily of G protein-coupled receptors characterized by seven transmembrane helices (TM1 to TM7) connected by three ex-
tracellular and three intracellular loops. The third intracellular loop and TM6 of TSHR appear to be hot spots for gain-of-function mutations (19, see TSH Receptor Mutation Database II, http://www.uni-leipzig.de/~innere/tsh/index.php). In the G protein-coupled receptor family, these domains may be particularly important for the interaction of the receptor with the G protein and its activation. A tightly packed hydrophobic cluster between the intracellular halves of TM5 and TM6 is critical for stabilizing the inactive receptor conformation [21]. Constitutive activity of mutant receptors is proposed to occur by disruption of the hydrophobic bonds between these residues and subsequent conformational changes of TM5 and TM6 in the plasma membrane, thereby enabling G protein to couple [22–26].

In this report, we describe the clinical and molecular findings in a Japanese family with hereditary nonautoimmune hyperthyroidism due to the novel Asp617Tyr TSHR mutation in the third intracellular loop of the TSHR.

Materials and Methods

Thyroid function analysis and thyroid volume

Concentrations of serum TSH, free thyroxine (FT4), and free triiodothyronine (FT3) were measured with enzyme immunoassays (AxSYM TSH, AxSYM FT4, and AxSYM FT3, respectively, Abbott Japan Co., Tokyo, Japan). Thyrotropin-binding inhibitory immunoglobulins (TBII) activity was measured with a commercial enzyme-linked immunoassay kit (RSR Ltd., Cardiff, UK). Thyroid-stimulating antibodies (TSAb) were measured by a commercial radioimmunoassay kit (Yamasa Co., Chiba, Japan). Thyroid volume was measured using ultrasound diagnostic equipment, as reported previously [27]. The estimated thyroid volume was calculated by the following equation: thyroid volume = 0.7 × (length × height × width) in both lobes of the thyroid gland.

DNA sequencing

Genomic DNA was extracted from peripheral leukocytes obtained from the patients and their family members. Exon 10 of the TSHR encoding the entire intracellular and transmembrane regions and part of the proximal extracellular domain of the TSHR was amplified with one set of primers by the polymerase chain reaction. Conditions were as follows: initial denaturation for 2 min (94°C), followed by 30 cycles of denaturation for 30 s (94°C), annealing for 30 s (55°C), and elongation for 90 s (72°C) with a final elongation step of 7 min (72°C). The forward primer was 5'-TAG GCT CAA GCA ATC CAC CTG-3'. The reverse primer was 5'-GTG TCA TGG GAT TGG AAT GC-3'. Direct sequencing of PCR products was performed using the Bigdye® terminator v1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA), and an automatic ABI 310 sequencer (Applied Biosystems), using either the same primers or internal primers (5'-ACT GTC TTT GCA AGC GAG TT-3' as a forward primer and 5'-GTC CAT GGG CAG GCA GAT AC-3' as a reverse primer). The present study was approved by the ethics committee of Kuma Hospital, and informed consent was obtained from the patients and their family members for the use of samples for research purposes.

Site-directed mutagenesis and functional expression of human TSHR

The full-length human TSHR complementary DNA (cDNA) in expression vector pCR 3 [28] was used as a template for mutagenesis. The Asp617Tyr and Asp619Gly TSHRs were generated using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. Sequences of mutated TSHR were verified as mentioned above. These plasmids were stably transfected into Chinese hamster ovary (CHO) cells in Ham’s F12 medium with 5% fetal bovine serum and the appropriate antibiotics using Lipofectin reagent (Invitrogen Life Technologies, Carlsbad, CA). Surviving cells were selected with 400 mg/L G418 (Geneticin, Sigma, St Louis, MO). A bulk of G418-resistant colonies was used for the subsequent studies.

Binding assays

125I-labeled bovine (b) TSH from a TRAb kit (Cosmic, Tokyo, Japan) was used in the binding studies. Cells (2 × 10^5/well) were seeded in 24-well culture plates, cultured overnight, and subjected to hormone binding studies, as described previously [29]. TSH binding was determined by lysing the cells with 1 mL 1% SDS and counting the radioactivity. The total num-
number of receptors expressed at the membrane surface, and dissociation constants (kD) were calculated by Scatchard analysis.

Cellular cyclic adenosine monophosphate (cAMP) and Inositol 1,4,5-triphosphate (IP3) measurements

For cAMP measurements, cells were seeded in 24-well culture plates (2 × 10^5/well) and cultured overnight. After washing with phosphate buffered saline (PBS), the cells were incubated for 60 min at 37°C in NaCl (–) Hank’s balanced salt solution (hypotonic HBSS) with 0.5 mmol/l 3-isobutyl-1-methylxanthine, 20 mmol/L HEPES pH 7.4, 0.4% bovine serum albumin (BSA) in the absence or presence of 10^{-7} M bovine (b) TSH. Cellular cAMP concentrations were determined with a commercial RIA kit (Yamasa Shoyu, Tokyo, Japan).

For IP3 measurements, cells in 6-well culture plates (2 × 10^5/well) grown overnight were washed with PBS and incubated for 1 min at 37°C in hypotonic HBSS with 25 mmol/L LiCl, 20 mmol/L HEPES pH 7.4, 0.4% BSA in the absence or presence of 10^{-7} M bTSH. The reaction was terminated by adding 10% KClO_4, and the mixture was kept on ice for 30 min and then neutralized with ice-cold 1.53 mol/L KOH, 75 mmol/L HEPES for 30 min. The samples were centrifuged at 2000 g for 15 min at 4°C to remove KClO_4 precipitate. The supernatant samples were assayed for IP3 accumulation using [3H] D-myo-inositol 1,4,5-trisphosphate assay (Amersham Pharmacia, Little Chalfont, Buckinghamshire, England).

Case Report and Results

Two cousins aged 20 and 21 years consulted our hospital with symptoms of hyperthyroidism, including heat intolerance, palpitation, and weight loss. Thyroid function tests showed that both probands, who are members 1 and 5 in the pedigree (Fig. 1A), had elevated levels of FT3 and FT4 and suppressed TSH levels with negative TBII or TSAb (Table 1). Ultrasoundography of the neck revealed diffuse goiters with thyroid volumes of 22.4 ml (member 1) and 34.7 ml (member 5) (Table 1). The radioiodine uptake of member 1 was 88.9% in 24 hours, and that of member 5 was 6.4% in 3 hours. Both were given 15 mg of methimazole daily. Despite normalization of serum FT4 levels, a whole-body urticarial rash developed in member 1 and liver dysfunction developed in member 5, at 1 month and 3 weeks, respectively, after the start of treatment. While methimazole was discontinued, both patients refused to undergo the thyroid ablation by

Fig. 1. A. Pedigree of the investigated family. Filled circles and squares represent affected members harboring the Asp617Tyr TSHR germline mutation. Open circles and squares represent unaffected members without the TSHR germline mutation. B. Sequencing analysis of TSHR exon 10 in genomic DNA extracted from peripheral blood leukocytes. A heterozygous guanine-to-thymine point mutation was identified at nucleotide position (nt.) 1849 in member 1 (a) and in a wt sequence in the father of member 1 (b).
surgery or radioiodine. Both patients were treated with potassium iodine that includes 38 mg of inorganic iodine daily. Seven months after the start of inorganic iodine treatment, thyroid function tests showed subclinical hyperthyroidism in both patients (TSH: 0.005 mIU/l; FT4: 1.34 ng/dl; FT3: 2.82 pg/ml in member 1; and TSH: <0.003 mIU/l; FT4: 1.37 ng/dl; FT3: 3.43 pg/ml in member 5). There was no tendency toward progressive enlargement of the goiter.

In the family history, member 6 (the mother of member 5) had been admitted to a local hospital because of congestive heart failure just before the diagnosis of hypothyroidism. Then she had undergone near-total thyroidectomy at the age of 48 years as a diagnosis of Graves’ disease and now received 100 µg of L-thyroxine daily. Other family members showed no symptoms of thyroid disease, but subsequent thyroid function tests revealed subclinical hyperthyroidism in members 2, 4, and 3 (Table 1). The grandfather died of a stroke at the age of 75 years. No serum anti-thyroglobulin antibodies or anti-thyroid peroxidase antibodies were found in any family members.

Table 1. Thyroid function analysis and thyroid volume

<table>
<thead>
<tr>
<th>Member number</th>
<th>Age (years)</th>
<th>FT4 (ng/dl)</th>
<th>FT3 (pg/ml)</th>
<th>TSH (µIU/ml)</th>
<th>TBII (%)</th>
<th>TSAb (%)</th>
<th>RAIU (%)</th>
<th>Thyroid volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>21</td>
<td>2.73</td>
<td>6.22</td>
<td>&lt;0.005</td>
<td>6.4</td>
<td>108</td>
<td>88.9*</td>
<td>22.4</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>1.25</td>
<td>3.39</td>
<td>0.009</td>
<td>8.7</td>
<td>NT</td>
<td>9.1</td>
<td>11.6</td>
</tr>
<tr>
<td>3</td>
<td>55</td>
<td>1.27</td>
<td>2.95</td>
<td>&lt;0.003</td>
<td>7.7</td>
<td>131</td>
<td>5.8</td>
<td>11.2</td>
</tr>
<tr>
<td>4</td>
<td>24</td>
<td>1.36</td>
<td>2.83</td>
<td>&lt;0.003</td>
<td>6.3</td>
<td>169</td>
<td>13.9</td>
<td>27.7</td>
</tr>
<tr>
<td>5</td>
<td>20</td>
<td>2.42</td>
<td>5.37</td>
<td>&lt;0.003</td>
<td>5.4</td>
<td>107</td>
<td>6.4</td>
<td>34.7</td>
</tr>
<tr>
<td>6**</td>
<td>48</td>
<td>3.43</td>
<td>7.31</td>
<td>NT</td>
<td>29.7</td>
<td>212</td>
<td>NT</td>
<td>NT</td>
</tr>
</tbody>
</table>

NT, not tested
Normal levels: FT4, 0.70–1.60 ng/dl; FT3, 1.70–3.70 pg/ml; TSH, 0.30–5.00 µIU/ml; TBII, <15%; TSAb, <180% RAIU; Radioiodine uptake in 3 hours, normal range 5.6–15.8% (* in 24 hours, normal range 10–40%)

**; just before surgery

Identification of TSHR gene mutation

Sequence analysis of genomic DNA extracted from white blood cells in members 1 and 5 showed an identical heterozygous guanine to thymine transversion at position 1849 (Fig. 1B(a) and not shown). This heterozygous mutation results in an amino acid substitution of aspartic acid for tyrosine at codon 617 in the third intracellular loop of the TSHR. Screening for the presence of the TSHR mutation was carried out for other available family members. This mutation was found in both mothers (members 3 and 6) and their sister (member 2) and brother (member 4); however, it was absent in their unaffected fathers and grandmother (Fig. 1B(b)).

Functional studies of the novel mutant receptors

Functional studies of the novel TSHR mutation were performed in CHO cells stably transfected with either wild-type (wt) or mutated TSHR. Further validation of the assay was carried out with Asp619Gly TSHR, another known constitutively activating TSHR [30]. Cell surface expression of Asp617Tyr and Asp619Gly TSHRs was reduced by approximately 40% compared with that of wt TSHR (Table 2). The mutated receptors displayed similar (Asp617Tyr) or slightly higher (Asp619Gly) affinity for bTSH compared with wt TSHR (Table 2).

Basal values of cAMP accumulation of Asp617Tyr and Asp619Gly TSHRs were approximately 40% compared with that of wt TSHR (Table 2). Basal values of cAMP accumulation of Asp617Tyr and Asp619Gly TSHRs were approximately 2.5-fold that of wt TSHR, while TSH-induced cAMP production was not affected by either mutated TSHR (Table 2). No constitutive activation of IP3 was demonstrated by either mutated TSHR (Table 2). The binding capacity values ruled out overexpression of the mutated receptors as a possible reason for the increased basal cAMP accumulation.

Discussion

We report on a Japanese family with nonautoimmune hyperthyroidism caused by a novel TSHR germ-line mutation. The Asp617Tyr is located in the third
intracellular loop of the TSHR and causes constitutive activation of the cAMP pathway. This mutation was present in all affected members with subclinical to overt hyperthyroidism but was absent in other members with normal thyroid function. A delayed and progressive onset of hyperthyroidism with mild clinical manifestations was very often observed in the previous cases with inherited mutation of the TSHR gene [1, 4, 12, 14, 17]. In this family, one mother harbored this TSHR mutation without clinically overt symptoms to date. Therefore, it is likely that the grandfather might harbor this mutation but showed no history of hyperthyroidism throughout his life. While previous reports showed that nonautoimmune hyperthyroidism was complicated with chronic thyroiditis in several cases [8, 14, 31], our data indicate that the clinical picture of the affected member 6 was complicated by autoimmune Graves’ disease.

Constitutively activating TSHR germline mutations have been predominantly identified in TMs but have also been identified in connecting intracellular and extracellular loops (Table 3). The known constitutively activating mutations in the third intracellular loop include several substitutions at codon positions 619 and 623 [30, 32, 33], and a deletion spanning 9 amino acids at codons 613–621 [5]. All but one of these mutations

### Table 2. Summary of TSH binding and cAMP and IP3 accumulations in bulk culture of CHO cells stably expressing the wt or mutated TSHR.

<table>
<thead>
<tr>
<th>TSHR cDNAs</th>
<th>TSH bindinga</th>
<th>cAMP (pmol/well)a</th>
<th>IP3 (pmol/well)a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>receptor number (/cell)</td>
<td>kD (pM)</td>
<td>basal concentration</td>
</tr>
<tr>
<td>wt</td>
<td>32,000 ± 3,000</td>
<td>850 ± 87</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>Asp617Tyr</td>
<td>19,000 ± 4,359b</td>
<td>650 ± 100</td>
<td>4.8 ± 0.3c</td>
</tr>
<tr>
<td>Asp619Gly</td>
<td>20,000 ± 2,646c</td>
<td>550 ± 70d</td>
<td>5.1 ± 0.7c</td>
</tr>
</tbody>
</table>

a, mean ± S.D. (n = 3)  
b, p = 0.01; c, p = 0.0065; d, p = 0.0095; e, p = 0.0001; f, p = 0.0016, compared with wt.

### Table 3. Characteristics of Hereditary Hyperthyroidism due to TSHR Mutations.

<table>
<thead>
<tr>
<th>Cases</th>
<th>TSHR mutation</th>
<th>Location of mutation</th>
<th>Activation of cAMP/IP3s</th>
<th>Earliest onset of hyperthyroidism</th>
<th>Goiter</th>
<th>Therapy</th>
<th>Surgery/Radioidine</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Gly 431 Ser</td>
<td>TM1</td>
<td>+/-</td>
<td>3 years</td>
<td>+</td>
<td>+/-</td>
<td>(9)</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Gly 431 Ser</td>
<td>TM1</td>
<td>+/-</td>
<td>5 years</td>
<td>–</td>
<td>+/-</td>
<td>(13)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Met 463 Val</td>
<td>TM2</td>
<td>+/-</td>
<td>2 years</td>
<td>+</td>
<td>+/-</td>
<td>(8)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Met 463 Val</td>
<td>TM2</td>
<td>+/-</td>
<td>2 years</td>
<td>–</td>
<td>+/-</td>
<td>(10)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Ser 505 Asn</td>
<td>TM3</td>
<td>+/-/ND</td>
<td>11 years</td>
<td>+</td>
<td>+/-</td>
<td>(17)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Ser 505 Arg</td>
<td>TM3</td>
<td>+/-</td>
<td>childhood</td>
<td>+</td>
<td>+/-</td>
<td>(4)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Val 509 Ala</td>
<td>TM3</td>
<td>+/-/ND</td>
<td>10 years</td>
<td>+</td>
<td>+/-</td>
<td>(18)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Val 509 Ala</td>
<td>TM3</td>
<td>+/-/ND</td>
<td>4 years</td>
<td>+</td>
<td>+/-</td>
<td>(1)</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Ile 568 Val</td>
<td>EC2</td>
<td>+/-</td>
<td>16 years</td>
<td>+/-</td>
<td>+/-</td>
<td>(12)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Val 597 Phe</td>
<td>TM5</td>
<td>+/-/ND</td>
<td>5 years</td>
<td>+</td>
<td>+/-</td>
<td>(16)</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Ala 623 Val</td>
<td>IC3</td>
<td>+/-</td>
<td>3.5 weeks</td>
<td>–</td>
<td>+/-</td>
<td>(3)</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Met 626 Ile</td>
<td>TM6</td>
<td>+/-/ND</td>
<td>1 month</td>
<td>+</td>
<td>ND</td>
<td>(15)</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Leu 629 Phe</td>
<td>TM6</td>
<td>+/-</td>
<td>2 years</td>
<td>+</td>
<td>+/-</td>
<td>(5)</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Phc 631 Ser</td>
<td>TM6</td>
<td>+/-</td>
<td>10 years</td>
<td>+</td>
<td>+/-</td>
<td>(14)</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Thr 632 Ile</td>
<td>TM6</td>
<td>+/-</td>
<td>7 month</td>
<td>ND</td>
<td>+/-</td>
<td>(7)</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Pro 639 Ser</td>
<td>TM6</td>
<td>+/-/ND</td>
<td>5 years</td>
<td>+</td>
<td>+/-</td>
<td>(6)</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>Asn 650 Ser</td>
<td>TM6</td>
<td>+/-/ND</td>
<td>5 years</td>
<td>+</td>
<td>+/-</td>
<td>(4)</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>Cys 672 Tyr</td>
<td>TM7</td>
<td>+/-/ND</td>
<td>14 years</td>
<td>+</td>
<td>+/-</td>
<td>(1)</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>Asp 617 Tyr</td>
<td>IC3</td>
<td>+/-</td>
<td>20 years</td>
<td>+</td>
<td>+/-</td>
<td>present case</td>
<td></td>
</tr>
</tbody>
</table>

TM, transmembrane helix; EC, extracellular loop; IC, intracellular loop; ND, not described
have been identified in toxic thyroid nodules as somatic mutations [5, 30, 32, 33]. The residues at codons 619 and 623 are highly conserved in the corresponding regions of other G protein-coupled receptors [23, 34]. The relative importance of distinct amino acids at codons 613 to 621 was clarified by deletion studies of several amino acids. Deletions at codons 619 and 613–617 resulted in ligand-independent receptor activation; however, deletions at codons 615 and 616 did not significantly affect the functional properties of the receptor [34]. These findings imply that the deletion or any change at codon 613, 614, or 617 may lead to constitutive activation of the receptor, despite nonconserved residues in the corresponding region of other G protein-coupled receptors [23, 34]. In our study, the Asp617Tyr mutation resulted in constitutive activation of the cAMP pathway, which agreed with previous reports that most of mutations activated the cAMP pathway but not the inositol phosphate cascade (Table 3).

The affected members in this family showed an adult-onset overt hyperthyroidism with onset age greater than in previously reported patients with hereditary nonautoimmune hyperthyroidism (Table 3). In addition, the hyperthyroidism in the affected members was well controlled with inorganic iodine. These features raise the possibility that the relative TSHR activity with Asp617Tyr mutation might be lower than that with other mutations. Comparison of the specific constitutive activity index (basal cAMP/receptor number) showed that the activities of Asp619Gly and Ala623Val TSHRs in the third intracellular loop were approximately one sixth of those of Thr632Ile and Cys672Tyr TSHRs in TM6 and TM7, respectively [35]. The constitutively activating TSHR mutations generally exhibit reduced surface expression as compared with wt TSHR, because of interference with correct folding and trafficking of the receptor to the plasma membrane [36]. Because the expression levels and basal cAMP levels of Asp619Gly TSHR are almost equivalent between stably transfected CHO cells and transiently transfected COS-7 cells [30], it seems that Asp617Tyr TSHR is a relatively weak stimulator of cAMP production.

In the present study, we have identified a novel Asp617Tyr TSHR mutation, which is located in a non-conserved residue in the third intracellular loop of the TSHR but resulted in constitutive cAMP activation. Careful follow-up will be necessary to define whether normal thyroid function can be maintained with inorganic iodine for prolonged periods in the two probands and whether overt hyperthyroidism will develop in other family members in the future. If they show any tendency such as thyroid enlargement or resistance to iodine therapy, ablation therapy should be recommended without delay.

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