A Japanese family with nonautoimmune hyperthyroidism caused by a novel heterozygous thyrotropin receptor gene mutation

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BACKGROUND: Hyperthyroidism caused by activating mutations of the thyrotropin receptor gene (*TSHR*) is rare in the pediatric population.

METHODS: We found a Japanese family with hyperthyroidism without autoantibody. DNA sequence analysis of *TSHR* was undertaken in this family. The functional consequences for the Gs-adenylyl cyclase and Gq/11-phospholipase C signaling pathways and cell surface expression of receptors were determined *in vitro* using transiently transfected human embryonic kidney 293 cells.

RESULTS: We identified a heterozygous mutation (M453R) in exon 10 of *TSHR*. In this family, this mutation was found in all individuals who exhibited hyperthyroidism. The results showed that this mutation resulted in constitutive activation of the Gs-adenylyl cyclase system. However, this mutation also caused a reduction in the activation capacity of the Gq/11-phospholipase C pathway, compared with the wild type.

CONCLUSION: We demonstrate that the M453R mutation is the cause of nonautoimmune hyperthyroidism.

Thyrotropin (TSH) receptor (TSHR) is a plasma membrane G protein–coupled receptor (GPCR), a member of a large superfamily of GPCRs (1). This receptor consists of seven transmembrane domains (TMDs) and a large extracellular domain (ECD) that mediates the effect of TSH in thyroid development, growth, and hormone synthesis (1,2). TSH binds the TSHR and activates two signal transduction pathways: the Gs/adenylyl cyclase and Gq-phospholipase C pathways (2,3). The Gs/adenylyl cyclase system is thought to be important for most TSH-induced effects on the growth and the function of the thyroid gland (1,2). Moreover, it has been reported that the Gq/11-phospholipase C pathway is involved in thyroid hormone synthesis and growth of the thyroid gland (3).

Activating germline mutations of *TSHR* cause nonautoimmune sporadic congenital hyperthyroidism or familial nonautoimmune hyperthyroidism (4–13). To date, gain-of-function mutations in TMD 1, 2, 3, 5, 6, and 7 and in the ECD have been reported (4–13). These mutations have been shown to activate the Gs/adenylyl cyclase system in a ligand-independent manner (4–13).

In this study, we report a Japanese family with nonautoimmune hyperthyroidism caused by a gain-of-function mutation (M435R) in TMD2. An *in vitro* study demonstrated that M453R causes constitutive activation of the Gs/adenylyl cyclase pathway in the absence of a ligand, but it also results in loss of the activating function of the Gq/11-phospholipase C pathway.

CASE REPORTS

The pedigree of the proband and her family is shown in **Figure 1a**. The proband is denoted by the arrow. Family members with hyperthyroid who were subjected to gene analysis are shown by half solid squares and circles.

Case 1

The proband (a younger sister) was delivered at 38-wk gestation by normal vaginal delivery as the second child of unrelated Japanese parents. Her birth weight was 2,482 g, length was 45.8 cm, and head circumference was 35 cm. Because her mother had been diagnosed as having nonautoimmune hyperthyroidism 2 y before pregnancy of case 1, we tested thyroid function in cord blood at birth. The results were as follows: levels of FT3, FT4, and TSH in cord blood were 1.23 pg/ml, 0.75 ng/dl, and 8.789 μU/ml, respectively. At the age of 1 mo, she was observed to have tachycardia with a heart rate of 150 beats/min and poor body weight gain (body weight was 3,282 g). She did not have a goiter or exophthalmos. Laboratory tests confirmed hyperthyroidism with a TSH level of <0.03 μU/ml (normal range: 0.4–4), FT4 level of 3.12 ng/dl (normal range for age: 0.8–1.9), and FT3 level of 8.36 pg/ml (normal range for age: 1.6–4.7). No TRAb or thyroid stimulating antibody (TSAb) was identified in the patient. Based on these findings, she was also diagnosed as having nonautoimmune hyperthyroidism, and treatment with thiamazole (2 mg/d) was initiated. After 3 wk of thiamazole treatment, a euthyroid state was achieved. Thyroid hormone levels continued to fluctuate within the normal range; however, reduction of thiamazole resulted in the elevation of

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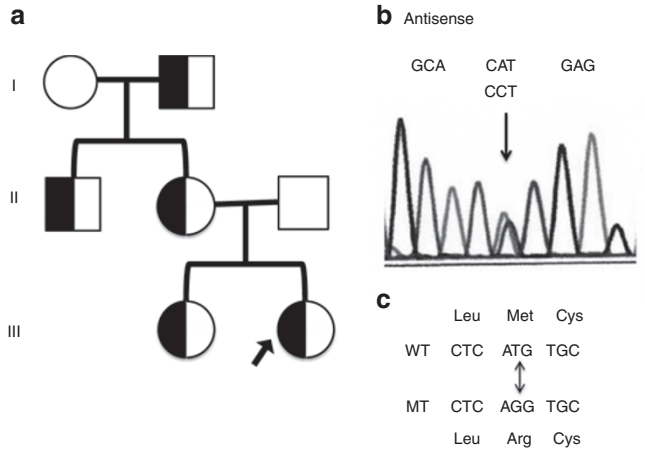

Figure 1. Analysis of *TSHR* in the family. (**a**) Pedigree of the family. The arrow indicates the proband. Squares and circles indicate males and females, respectively. Half solid squares and circles indicate the members who were diagnosed as having hyperthyroidism and subjected to gene analysis. (**b**) Sequencing of exon 10 of thyrotropin receptor (*TSHR*) identified both C and A nucleotides, denoted by the arrow. Note that this sequence is an antisense strand. (**c**) This base change results in substitution of arginine for methionine as a heterozygous state.

thyroid hormone and suppression of TSH levels. Goiter was noticed at the age of 2.5 y at regular evaluation. At the age of 5 y, thyroid ultrasonography showed diffuse enlargement of the thyroid gland, with a total volume of 9.7 cm^3 (normal: 3.3 ± 1.0 cm^3 for her age) (14) and high blood flow in the thyroid tissue. Now aged 6 y, she continues to receive thiamazole at 10 mg/d. Her height is 111.3 cm (−1.2 SD for a normal Japanese girl), and her BMI is 13.8 kg/m^2 (−1.18 SD for a normal Japanese girl).

Case 2

Case 2 is the mother of case 1. She delivered the first child (case 3) at the age of 21 y. She had complaints of headache and easy fatigue at the age of 24 y. At this time, goiter was noticed by physical examination. Thyroid function test showed elevated FT3 and FT4 and undetectable TSH in the absence of autoantibodies (FT4 6.24 pg/ml and FT4 2.24 ng/ml). Ultrasonography revealed a diffuse enlargement of thyroid gland (total volume was 44.2 cm^3) (normal range: 10.9 ± 2.5 cm^3), and ^{123}I uptake was high (40% at 24 h, normal range: 15–25%). Her father and younger brother were also diagnosed as having hyperthyroidism; however, detailed clinical data were not available. Based on these findings, she was diagnosed as having nonautoimmune hyperthyroidism, and administration of thiamazole was initiated. Clinical symptoms were improved; however, urticaria developed after 1 mo of treatment. Thereafter, her medication was changed to propylthiouracil (600 mg/d). Two years after diagnosis of hyperthyroidism, she was pregnant with the second child. During pregnancy, her thyroid function was stabilized with treatment of propylthiouracil at 150 mg/d.

Case 3

Case 3 is the elder sister of the proband. She was born at the 40th wk of gestation following an uncomplicated delivery. Her birth weight was 3,855 g, her length was 52 cm, and head circumference was 37.5 cm. She was born 3 y before diagnosis of hyperthyroidism in her mother was done. Neonatal screening for hypothyroidism based on TSH measurement did not detect any abnormality. Because of hyperthyroidism in her younger sister, mother, and other family members (**Figure 1a**), her thyroid function was evaluated at the age of 5 y 9 mo. Until then, her general health was good, and her physical and neurological development were normal. Her height was 115 cm (±0 SD for a normal Japanese girl), and her BMI was 15.1 kg/m^2. Hormone measurement showed elevated thyroid hormone and suppressed TSH levels (FT3 = 5.96 pg/ml, FT4 = 2.00 ng/dl, and TSH < 0.05 μU/ml) in the absence of thyroid autoantibodies. Physical examination showed no goiter, but her heart rate was slightly elevated (90 beats/min). Because her general condition was good, she was followed without medication. However, because her thyroid hormone and TSH levels remained high and suppressed, respectively, administration of thiamazole (15 mg/d) was started at the age of 6 y 3 mo. Because systemic urticaria developed 1 mo after the initiation of thiamazole, propylthiouracil was substituted for thiamazole. A goiter gradually became evident. At the age of 9 y, thyroid ultrasound revealed a diffuse goiter with a total volume of 8.1 cm^3 (normal: 4.9 ± 1.0 cm^3 for her age) (14) and high blood flow in the thyroid tissue. She is now 10 y old, and administration of propylthiouracil (300 mg/d) is being continued.

RESULTS

Mutation Screening in *TSHR*

In the proband, her younger sister, her mother, her maternal grandfather, and her maternal uncle, a heterozygous c. 1358 T>G mutation was identified. As a result of this change, methionine at codon 453 was changed to arginine (M453R) in exon 10 of *TSHR* (**Figure 1b**,**c**). This missense mutation was present in TMD 2. The proband's father did not have this nucleotide change. At the same codon of 453, an M453T mutation was previously reported in two sporadic patients with severe nonautoimmune hyperthyroidism (6,7).

Cell Surface Expression of M453R

Western blotting demonstrated that both M453R and M453T expression were similar to the wild-type (WT) receptor (**Figure 2a**). Further quantification of cell surface expression by flow cytometry demonstrated a reduced expression level compared with WT (**Figure 2b**) (WT: 100.23 ± 10.2%, M453R: 80.6 ± 19.5%, and M453T: 61.2 ± 18.9%). The reduced cell surface expression of M453T was in agreement with the result previously reported (6).

Functional Consequence on Gs-Adenylyl Cyclase and Gq/11-Phospholipase C Signaling Pathways

Despite the lower expression level, the basal activity of both M453R (853 ± 110%) and M453T (923 ± 89%) was significantly increased compared with that of the WT (**Figure 3a**). The degree of constitutive activation of M453T was comparable with that of M453R. When stimulated with 100 mIU/ml of TSH, the

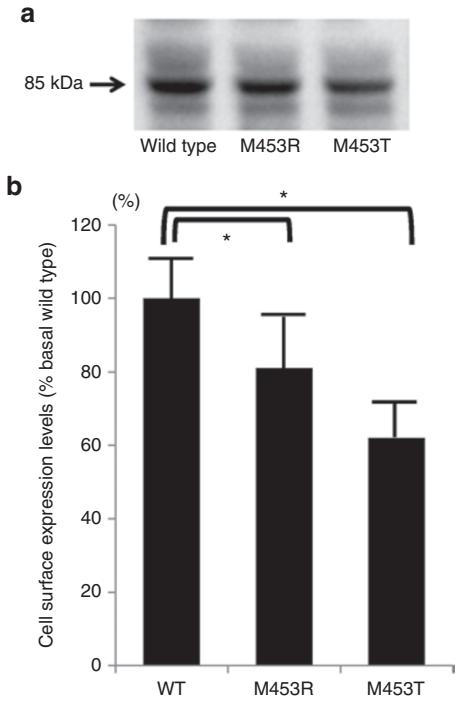

Figure 2. Cell surface expression of mutant *TSHR*. (**a**) Western blotting showed that protein expression levels of both M453R and M453T in whole cells were similar to those of wild type (WT). (**b**) Cell surface expression after transfection of empty pcDNA3 plasmid, WT, M453R, and M453T was measured by flow cytometry (*$P < 0.05$). Reduced expression of M453R and M453T compared with WT thyrotropin receptor (TSHR) was observed. The data represent the mean ± SEM.

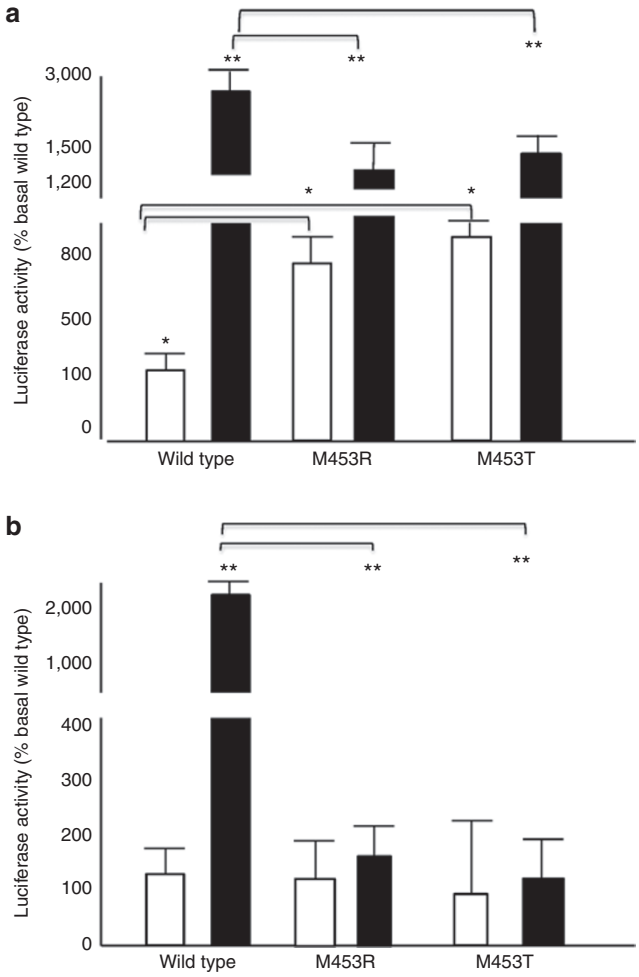

Figure 3. Functional analysis of mutant *TSHR*. (**a**) Activation of Gs-adenylyl cyclase was determined 48 h after transfection. Cells were stimulated with bovine thyrotropin (TSH). Basal activity is indicated by white bars; activity after stimulation with 100 mU bovine TSH is indicated by black bars. The basal activity of the wild type (WT) was set as 100%. The data represent the mean ± SEM. The basal activity of both M453R and M453T was significantly increased compared with that of WT, indicating that these two mutants are constitutively active (*$P < 0.05$; **$P < 0.01$). (**b**) Activation of the Gq/11-phospholipase C signaling pathway was determined 48 h after transfection. Basal activity is indicated by white bars; and activity after stimulation with 100 mU bovine TSH is indicated by black bars. The basal activity of the WT was set as 100%. The data represent the mean ± SEM. When M453R and M453T were stimulated by 100 mIU/ml of bovine TSH, both mutants completely lost activation capacity compared with WT (**$P < 0.01$).

response of each mutant was significantly reduced compared with WT (WT = 2,763 ± 125%, M453R = 1,324 ± 637%, and M453T = 1,466 ± 324%).

Next we studied the basal and TSH-stimulated activation of the Gq-phospholipase C signal pathway (**Figure 3b**). Basal activation of M453R and M453T was not significantly different from that of the WT. When each mutant was stimulated by 100 mIU/ml of TSH, both mutants completely lost their activation capacity compared with the WT (**Figure 3b**).

DISCUSSION

To date, ~30 constitutively activating *TSHR* mutations have been reported in nonautoimmune hyperthyroidism (4–13,15–19). Most of the activating mutations are present in TMD 1, 2, 3, and 5. Our mutation was also located in TMD2. Previously, two *TSHR* germline activating mutations have been found in TDM2 (M453T and M463V) (6,7,9,10). However, because there is no hot spot for the activating mutation, analysis of *TSHR* in each individual with nonautoimmune hyperthyroidism is necessary.

Two points regarding the clinical phenotypes and the genotypes of mutations of *TSHR* are pointed out in this study. First, regarding goiter, its onset is variable even in cases and families harboring the same mutations of *TSHR* (10,11,16,18,19). In some patients, a goiter develops before the onset of clinical findings of hyperthyroidism (15); however, in other patients, the manifestation of hyperthyroidism precedes the onset of goiter (7,8,11,12,16). In the siblings of our family, goiter was observed at 2–3 y of age after the diagnosis of hyperthyroidism. Fuhrer *et al.* (20) reported that follicular cell growth in the thyroid due to the constitutive activation of TSHR takes a longer period than stimulation of function. This may explain the later manifestation of goiter than that of hyperthyroidism in our siblings.

Second, hyperthyroidism in our familial cases was not severe. In the proband, poor body weight gain and tachycardia were noticed at 1 mo of age; however, hyperthyroidism was not severe. Based on the past history, her elder sister was likely to be asymptomatic, and her elevated thyroid hormone levels

were observed by blood examination at the age of 5 y. Their mother was diagnosed at the age of 24 y. In contrast to our familial cases, previously reported patients harboring M453T mutation showed severe hyperthyroidism from the fetal period (6,7). In one case, the patient exhibited goiter, tachycardia, and marked ocular symptoms at birth (6). Another patient showed hyperthyroidism from the fetal period, and thyroidectomy and radiotherapy were required to achieve a euthyroid state (7). These phenotypic differences may be due to the type of mutation in *TSHR*. However, an *in vitro* study showed that the degree of constitutive activation of the Gs-adenylyl cyclase signaling pathway of the two mutants was similar.

Winkler *et al.* (17) reported the clinical course and molecular mechanism in one patient with nonautoimmune hyperthyroidism caused by a C636W mutation of *TSHR*. The patient showed very mild symptoms and no goiter. In an *in vitro* study, the C636W mutant stimulated the Gs-adenylyl cyclase pathway in a constitutive manner; however, activation of Gq-phospholipase C stimulated by TSH was markedly reduced compared with the WT. Based on these findings, the authors suggested that the lack of goiter in their patient might be due to impairment of the Gq-phospholipase C signal pathway because Gq-phospholipase C is involved in thyroid hormone synthesis and growth of the thyroid gland as well as the Gs-adenylyl cyclase pathway(3). In this context, we compared the activation capacity of Gq-phospholipase C between M453R and M453T; however, both mutants had completely lost the capacity to activate this pathway. Therefore, our *in vitro* data show that the genotype alone could not explain the phenotypic severity of our familial cases with the M453T mutation. As suggested previously, other genetic and/or environmental factors are likely to influence the phenotype of nonautoimmune hyperthyroidism (10,11,18,19).

In conclusion, we identified a novel mutation of *TSHR* causing nonautoimmune hyperthyroidism.

METHODS

DNA Amplification and Sequence Analysis

Informed consent to participate in the study was obtained from the patients and/or their parents. The ethical committee of Hokkaido University School of Medicine approved this study. Genomic DNA was extracted from peripheral leukocytes, and *TSHR* was amplified by PCR as described previously (21). These PCR products were purified and directly sequenced with an automated sequencer (ABI Prism; Perkin-Elmer, Wellesley, MA).

Site-Directed Mutagenesis and Plasmid Construct

Human *TSHR* cDNA was inserted into a human expression vector (pcDNA3.1; Invitrogen, Carlsbad, CA). M453R identified in our study was created by site-directed mutagenesis using the PrimeSTAR mutagenesis kit (Takara, Tokyo, Japan). We also created M453T, which was previously reported in patients with nonautoimmune hyperthyroidism (6,7). A modified *TSHR* cDNA containing a FLAG epitope tag, YKDDDDK, inserted between amino acids 20 and 21 just after the signal peptide in the TSHR NH2-terminal region was constructed.

All constructs were confirmed by DNA sequencing before transfection.

Expression of TSHR in Human Embryonic Kidney 293 Cells

Human embryonic kidney (HEK) 293 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) with 10% heat-inactivated fetal bovine serum. Transient transfection was performed by using Lipofectamine 2000 (Invitrogen).

Western Blot Analysis

HEK-293 cells were plated into 75 cm^2 flasks, grown to 70% confluence, and transiently transfected with 10 μg WT or mutant FLAG-tagged TSHR cDNA. Forty-eight hours after transfection, crude cell extracts were obtained, and samples containing 50 μg of protein were separated by 10% SDS-polyacrylamide gel electrophoresis. The presence of the TSHR was detected with an anti-FLAG antibody and visualized by ECL Plus Western Blotting Detection Reagents (GE Healthcare, Little Chalfont, UK).

Fluorescence-Activated Cell Sorting Analysis

To quantify the extent of cell surface expression of TSHR, fluorescence intensity was measured using flow cytometry. HEK-293 cells grown in six-well plates were transiently transfected with 2 μg WT or mutant FLAG-tagged *TSHR* cDNA and 0.5 μg pAcGFP1-Mem (Clontech-Takara, Tokyo, Japan), which results in labeling of the plasma membrane. Forty-eight hours after transfection, cells were gently scraped with Accutase-Enzyme Cell Detachment Medium (eBioscience, San Diego, CA). Detached cells were washed with ice-cold PBS, and 10^6 cells were incubated for 20 min at 4 °C with anti-FLAG antibody diluted 1:500. Cells were washed with PBS and incubated for another 20 min with PE-conjugated Goat anti-mouse IgG diluted 1:250 (BioLegend, San Diego, CA) at 4 °C. Cells were washed with PBS and immediately analyzed on a BD FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Data were analyzed using Cellquest software (Becton Dickinson). All transfections were performed in triplicate, and the experiment was repeated three times. Cell surface expression levels of TSHR were expressed relative to the levels of green fluorescent protein to control for transfection efficiency.

Functional Characterization of Mutant TSHR

Activation of G protein–coupled signal transduction by TSHR (WT or mutant) was studied using luciferase assays: cAMP-response element (pGL4.29; Promega, Madison, WI) and nuclear factor AT response element (pGL4.30; Promega) were used to assess the Gs/adenylyl cyclase and Gq-phospholipase C pathway, respectively, as described in previous reports (21,22).

We seeded HEK-293 cells into 12-well plates and transfected the cells with 0.5 μg of each TSHR construct (WT or mutant) along with 0.5 μg of each reporter vector. Forty-eight hours after transfection, the medium was changed, and the cells were incubated with Dulbecco's modified Eagle's medium with and without bovine TSH (100 mU/ml) for 30 min (Sigma, St. Louis, MO). The assay was performed according to the manufacturer's protocol. Luciferase activity was normalized to β-galactosidase. All transfections were performed in triplicate, and the experiment was repeated three times. Values were represented as the mean ± SEM. Statistical analysis was carried out using the Mann–Whitney *U* test.

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