CLINICAL STUDY

A novel activating mutation in the thyrotropin receptor gene in an autonomously functioning thyroid nodule developed by a Japanese patient

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Abstract

Objective: A number of activating mutations of the thyrotropin receptor (TSHR) have been found in autonomously functioning thyroid nodules (AFTNs) in European patients. We aimed to study TSHR mutation in AFTNs in Japanese patients because no TSHR activating mutation has been found by previous incomplete studies.

Design: A typical AFTN developed in a 69-year-old Japanese woman was studied.

Methods: The entire exon 10 of the TSHR cDNA was sequenced. Functional studies were done by site-directed mutagenesis and transfection of a mutant construct into COS-7 cells.

Results: We identified a novel heterozygous TSHR gene mutation, Leu512→Arg (L512R; CTG→CCG), from the AFTN. The mutation was not detected in the adjacent normal thyroid tissue. COS-7 cells transfected with L512R mutant TSHR expression vector exhibited a 3.3-fold increase in basal cAMP level compared with that of cells transfected with wild-type TSHR DNA, confirming that the mutation was the direct cause of the AFTN.

TSHR activating mutations involving the third transmembrane helix reported to date are S505R/N and V509A as well as L512R. An *in vitro* site-directed mutagenesis study encompassing residues 505–513 revealed that mutations involving residues other than these three did not show constitutive activation.

Conclusion: This is the first TSHR activating mutation found in a Japanese patient, although true prevalence of TSHR activating mutations in AFTNs developed in Japanese patients remains to be elucidated. In addition, functional studies suggested that amino acid residues in the third transmembrane helix maintaining inactive conformation of the TSHR seem to be located on the same surface of the α -helix, possibly making interhelical bonds with another helix.

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Introduction

Autonomously functioning thyroid nodules (AFTNs) are a frequent cause (~30%) of hyperthyroidism in European populations, but are very rare in Japan (~0.3% of hyperthyroidism) (1). Further, a number of mutations of the thyrotropin receptor (TSHR) have been reported in AFTNs in Europeans (2–8). These mutant TSHRs showed high basal cAMP level without agonist stimulation when transfected into cells without TSHR. They constitutively activated adenylate cyclase and A-kinase and finally led to cellular hyperfunction and proliferation, as well as constitutively activated mutation of $G_{s\alpha}$, *gsp* (9). The frequency of TSHR activating mutations in AFTNs in European patients differed

between reports, but reached up to 80% when careful full-length sequencing was performed (7). Although it is unknown whether only TSHR or $G_{s\alpha}$ activating mutations can give rise to AFTN (10), it is believed that these mutations account for most AFTNs developed in European subjects. Some authors have speculated that these mutations, like *ras*, *gsp*, *ret* and *trk* mutations (11–13), might have been triggered by iodide deficiency due to unknown mechanism(s) (14).

In Japan, Takeshita *et al.* (15) and Tanaka *et al.* (16) found only one *gsp* mutation in a total of 45 AFTNs and toxic multinodular goiters (TMNGs). Murakami *et al.* (17) examined an AFTN and found another *gsp* mutation. However, no TSHR activating mutation has been detected to date in Japanese patients, in contrast to

European populations. Therefore, it has been suggested that there are no TSHR activating mutations in AFTNs in the Japanese population and that the etiology of AFTNs in Japanese subjects may be different from that in Europeans. However, there were serious methodological problems (short analyzed region and insensitive screening methods) especially in the work of Takeshita *et al.* (15), and the frequency of TSHR mutations would have been markedly underestimated.

We identified a novel, somatic and heterozygous mutation in the TSHR gene in an AFTN developed in a Japanese woman. Here, we report the first TSHR activating mutation to be found in a Japanese patient and discuss the methodological issues in detection of TSHR mutation as well as the functional significance of this novel mutation.

Subject and methods

Patient

A 69-year-old Japanese woman visited the thyroid clinic of Kyoto University Hospital for examination of a thyroid nodule. She had a nodular, elastic-firm, smooth-surfaced, clearly margined, well movable goiter measuring 3.2 cm × 2.5 cm in the right lobe. Ultrasonography showed a nodule with high echogenicity and high blood flow. The left lobe was atrophic. Laboratory examination revealed that she was mildly thyrotoxic; T₄, 166 µg/l (normal range, 50–110 µg/l); T₃, 1.87 µg/l (normal, 0.94–1.54 µg/l); free T₄, 24.1 ng/l (normal, 9.8–17.7 ng/l); free T₃, 5.23 ng/l (normal, 2.8–4.6 ng/l); TSH, <0.03 mU/l (normal, 0.3–3.9 mU/l); thyroglobulin, 137 µg/l (normal, <45 µg/l); and TRAb, 5.8% (normal, –10 to +10%). No abnormal findings were found on chemical or hematological examinations. ^{99m}Tc-pertechnetate thyroidal scintigraphy showed a 'hot nodule' in the right lobe with increased uptake (4.3% at 30 min; normal, 0.4–3.0% for the entire thyroid). The surrounding thyroid was completely suppressed. Under the diagnosis of typical AFTN, right hemithyroidectomy was performed. Histological examination showed clearly encapsulated follicular thyroid adenoma.

Extraction of RNA and genomic DNA, reverse transcription (RT), PCR of exon 10 of TSHR DNA, and direct sequencing

Informed consent was obtained from the patient for the use of samples for research purposes. Total RNA was extracted by the acid guanidinium–phenol–chloroform method from approximately 100 mg thyroid tissue using RNeasy B (Biotecx Laboratories, Houston, TX, USA) from the tumor and adjacent normal thyroid tissues. Genomic DNA was extracted from the peripheral blood cells as described previously (18). RT was performed on 2 µg total RNA (preheated at 70 °C for

10 min) in 40 µl reaction buffer (25 mM Tris–HCl (pH 8.3), 50 mM KCl, 2 mM dithiothreitol, and 5 mM MgCl₂) containing 30 pmol oligo(deoxythymidine)₁₅ primer, 20 U avian myeloblastosis virus reverse transcriptase XL (Life Sciences, Petersburg, FL, USA), and 40 U ribonuclease inhibitor (Toyobo, Osaka, Japan) at 42 °C for 40 min (19). For PCR amplification of a TSHR DNA fragment of 1445 bp in exon 10, which encodes the entire transmembrane portion and cytoplasmic tail, 4 µl RT reaction solution or 10 ng genomic DNA was incorporated in 100 µl PCR buffer (10 mM Tris–HCl (pH 8.5), 50 mM KCl, 1.5 mM MgCl₂) containing 2 U Taq polymerase (Takara, Tokyo, Japan) and 50 pmol each of oligonucleotide primers, 5'-CCTTGAGTCCTTGATGTGTAATG-3' (corresponding to nucleotides +885 to +907) and 5'-CCTTGAGTCCTTGATGTGTAATG-3' (+2308 to +2329). Samples were denatured for 5 min at 94 °C and then subjected to 35 cycles consisting of 1 min at 94 °C, 1 min at 58 °C, and 1 min at 72 °C. The last extension was carried out for 10 min. Purified ~1.4 kbp PCR products were subjected to direct sequencing with the PCR primers and primers derived from exon 10. Sequences from nucleotide +908 to +2307 of human TSHR DNA covering almost the entire exon 10, were determined in both orientations by direct sequencing with a GeneScan DNA sequencer 373A (Perkin-Elmer, Foster City, CA, USA) (18).

Construction of expression vectors, site-directed mutagenesis of the human TSHR DNA, transfection and assays

WT (wild-type) human TSHR cDNA in the pSG5 vector (Stratagene, La Jolla, CA, USA) under control of the SV40 promoter was constructed as reported previously (20). Expression vectors of mutant TSHRs were generated by site-directed mutagenesis using a Gene Editor kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. Mutations were confirmed by DNA sequencing of the final construct. Mutant plasmid DNA was purified by CsCl gradient ultracentrifugation.

Approximately 10⁷ COS-7 cells were transfected with 25 µg mutant or WT TSHR DNA or control vector pSG5 DNA by electroporation. When less of the WT TSHR DNA was applied, the total DNA used for transfection was adjusted to 25 µg by addition of pSG5 vector DNA to avoid changes in transfection efficiency and cell viability. Aliquots of the same batch of transfected cells were plated for TSH binding (~5 × 10⁵ cells/well in 6-well plates) and assays of cAMP and inositol phosphate (IP) production (~10⁵ cells/well in 24-well plates); medium was inositol-free in the latter assay and supplemented with 2.5 µCi/ml myo-[2-³H(N)]inositol (DuPont-NEN, Boston, MA, USA).

All assays were initiated simultaneously 48 h after transfection and after washing with assay buffer: Hanks' balanced salt solution containing 0.5% crystalline

bovine serum albumin (BSA), 20 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES)–NaOH, pH 7.4. [125 I]TSH binding was measured after incubation for 2 h at 22 °C in 1 ml NaCl-free assay buffer containing 222 mM sucrose, $\sim 100\,000$ c.p.m. [125 I]TSH (~ 40 μ Ci/ μ g) and 0 to 10^{-7} M unlabeled TSH. Total cAMP and IP levels were measured in the same wells after incubation for 1 h at 37 °C with 0.2 ml assay buffer containing 10 mM LiCl, 0.5 mM 3-isobutyl-1-methylxanthine, and 10^{-12} to 10^{-7} M TSH as noted. Total cAMP was measured by RIA, and IP formation was determined using anion exchange columns. TSH for functional assays and labeling was obtained from the NIH hormone distribution program (NIDDK-bTSH-I-1, 30 U/mg).

All assays were performed at least in triplicate, on at least two separate occasions with different batches of cells, and always included control cells transfected with WT DNA or pSG5 vector alone. LIGAND software was used to calculate the dissociation constant (K_d) and receptor density (B_{max}) values for TSH binding (21). K_d data were log-transformed, averaged and reconverted to calculate geometric means. A K_d confidence limit of 95% was obtained by log-transformation, calculating mean -1.96 S.D. and mean $+1.96$ S.D., and reversion (Table 1). Cyclic AMP and IP data are expressed as fold increase over basal in cells transfected with WT TSHR DNA in Table 1, and as fold increase over basal in cells transfected with the pSG5 vector alone in Figs 2 and 3.

To monitor transfection efficiencies, 0.1 μ g pSVGH was cotransfected with mutant or WT TSHR plasmid DNA, or control vector. Forty-eight hours after transfection, the medium was taken for RIA of human GH concentration. Data from transfectants in which transfection efficiencies differed more than 10% from the control were discarded.

To evaluate receptor density estimated by TSH binding experiments, flow cytometric analysis was performed with FLAG-tagged mutant receptors and anti-FLAG antibody. FLAG sequence (DYKDDDDK) was inserted between the 23rd and 24th amino acids of the wild-type TSH receptor construct by site-directed mutagenesis. FLAG-mutant receptors were constructed by ligation. Cell preparation and procedures were essentially the same as previously described (7, 22). Cells were incubated with 1:100 diluted anti-FLAG antibody and then with FITC-conjugated anti-mouse IgG. Relative expression level was the ratio between the fluorescence intensity of mutant transfectant and that of wild-type transfectant after subtracting the background of control cells (22).

Results

Detection of TSHR mutation

By sequencing the entire exon 10 of the TSHR gene, a novel heterozygous nucleotide substitution of T to C was

Figure 1 Sequences around codon 512 of the TSHR gene in the tumor and adjacent thyroid tissue. Antisense sequences are shown. Leu512 (CTG) was heterozygously substituted with Arg (CGG) in the tumor (left panel) but not in adjacent thyroid tissue (right panel). Sequences from peripheral blood cells were the same as the adjacent thyroid.

identified at nucleotide position +1535, which changed Leu512 to Arg in the third transmembrane helix in an AFTN developed in a Japanese woman (Fig. 1). No other nucleotide changes from the reported sequences except common polymorphisms (23–25) were found in the region from nucleotide +908 to +2307 containing most of exon 10. The L512R mutation was not detected in the adjacent normal thyroid tissue or peripheral blood cells from the patient.

Expression of L512R mutant

Expression vectors were constructed for the mutant (L512R) and WT TSHR and simultaneously transfected into COS-7 cells. As shown in Fig. 2A and Table 1, the L512R transfectant showed a 3.3-fold increase in basal cAMP level compared with that in the WT transfectant, confirming that the mutation was the direct cause of the AFTN in the patient. D633E, which was previously shown to be constitutively activated (20), showed a similar basal cAMP level to the L512R transfectant. However, the L512R transfectant did not respond to TSH-stimulation unlike D633E.

Binding experiments showed that the TSH K_d of L512R was similar to that of WT, but surface receptor density, calculated as B_{max} , was lower than that of WT (Table 1). The transfectant with 5 μ g WT TSHR and 20 μ g pSG5 DNA (WT(5)) showed a B_{max} not higher than that of L512R. The maximal cAMP response to TSH in WT(5) was higher than that in L512R. The receptor densities estimated from the TSH binding study were in good accordance with those determined by flow

Table 1 Characterization of mutant TSH receptors.

	WT	L512R	D633E	WT(5)
TSH binding				
K_d (pM)*	320 (127–804)	233 (104–522)	144 (46–455)	291 (116–731)
% B_{max} (/WT)**	100	52.4 $\pm$ 6.0	62.2 $\pm$ 3.5	47.9 $\pm$ 2.9
%Re Exp Level (/WT)***	100	56	65	51
cAMP increase				
%Basal (/WT basal)****	100	325 $\pm$ 6	333 $\pm$ 15	82 $\pm$ 8
Max Resp (/WT basal)*****	668 $\pm$ 79	328 $\pm$ 8	689 $\pm$ 29	515 $\pm$ 32
IP increase				
%Basal (/WT basal)	100	100 $\pm$ 6	92 $\pm$ 9	99 $\pm$ 9
Max Resp (/WT basal)*****	798 $\pm$ 32	102 $\pm$ 4	497 $\pm$ 63	384 $\pm$ 38

* Geometric mean. Values in parentheses are 95% confidence limits. ** WT B_{max} averaged 76.5 $\pm$ 16.2 fmol/well. *** Relative expression level estimated by flow cytometry using FLAG constructs. **** Basal cAMP level in the wild-type transfectant was 29.5 $\pm$ 2.1 pmol/well. ***** Maximal response at 10^{-7} M TSH. The mutant B_{max} , cAMP and IP data (mean $\pm$ S.E.M.) are expressed as percentages of WT TSHR values.

cytometric analysis using FLAG-tagged receptors and anti-FLAG antibody (Table 1).

Constitutive activation of the IP signal was not observed in the L512R transfectant similarly to D633E (Fig. 2B). WT(5) as well as D633E responded well to TSH with a maximal response half that observed in WT. However, no TSH-stimulated IP increase was observed in the L512R transfectant.

Characterization of mutants involving residues 505–513

In the third transmembrane helix Ser505 and Val509, as well as Leu512, were found to be mutated in human diseases (26–28). We constructed mutants by substituting one of the residues 505–513 with Ser, Ala or Arg, which were substituted in identified activating

mutations involving the third transmembrane helix (Fig. 3). Basal cAMP levels in S505R, V509A and L512R transfectants were higher than that in the WT transfectant, as reported previously (26, 27). Other mutants showed lower cAMP levels than WT. As most of the mutants showed lower B_{max} than WT and basal cAMP level was dependent on B_{max} , constitutive activation can be excluded when the basal cAMP level in the mutant transfectant is lower than that in the WT transfectant with a similar or lower B_{max} . Based on this comparison, no constitutive activation was observed in other mutants.

Discussion

We identified a novel activating TSHR mutation, L512R, in an AFTN developed in a Japanese patient.

Figure 2 Basal and TSH-stimulated accumulation of cAMP (A) and inositol phosphates (B) in COS-7 cells transfected 48 h earlier with 25 μ g each of control vector (pSG5), wild-type human TSHR DNA (WT), or TSHR DNA encoding L512R or D633E (positive control mutant used previously (20)) mutant. WT(5) shows transfectant with 5 μ g WT TSHR DNA and 20 μ g pSG5 vector DNA. Data are means $\pm$ S.E.M. of three experiments, and are expressed as fold increase over basal in cells transfected with the pSG5 vector alone. The cAMP level in the control transfectant was 11.2 $\pm$ 0.6 pmol/well.

Figure 3 Basal cAMP level of COS-7 cells transfected with TSHR mutant involving one of the amino acid residues 505–513. Values are expressed as fold (mean $\pm$ S.E.M.; $N = 6$) over basal in control transfectant (pSG5) in an experiment (basal cAMP level in the control transfectant was 10.9 ± 0.8 pmol/well). Another independent experiment showed similar results. K_d values for each mutant are not shown but ranged from 95 to 397 pmol/l. B_{max} values estimated from TSH binding are shown and expressed as a percentage of WT transfectant B_{max} . Dark grey columns indicate transfectants with B_{max} higher than that of 25 μ g WT transfectant (WT; 100%). Light grey columns indicate transfectants with B_{max} higher than that of 5 μ g WT transfectant (WT(5); 52%). White columns indicate transfectants with B_{max} higher than that of 1 μ g WT transfectant (WT(1); 19%). Cyclic AMP levels in mutant transfectants should be compared with the WT transfectant with the same color column (similar or lower receptor density).

This is the first activating TSHR mutation identified in a Japanese subject including both somatic and germline mutations.

Takeshita *et al.* (15) examined 38 AFTNs and seven TMNGs in Japanese patients. They found a TSHR gene alteration, $\Delta 619+T620S$ (deletion of three adenines) in only one AFTN. This mutant did not show constitutive activation in expression experiments so failing to confirm that this was a disease-causing mutation. They concluded that there was no constitutively activating mutation of the TSHR in AFTNs in the Japanese population. No confirmation study for Japanese population followed this entirely negative and discouraging conclusion. However, caution must be paid in interpreting their results as their methodology seemed to markedly underestimate the frequency of the TSHR mutation. The region in which they were able to analyze mutations was from nucleotide +1829 to +1955 (amino acid residues 610–652), shorter than 1/10 of the exon 10 coding region, and outside the region in which we identified the L512R mutation. They screened for mutations using SSCP (single strand conformation polymorphism), which is known to have low sensitivity (8), using only one protocol with one positive control mutant with eight nucleotide substitutions (5, 29). Direct sequencing of this region was performed in only 10 AFTNs, and no mutations were found. However, the presence of mutations in this

region could not be excluded using their method because of the possibility of contamination with DNA from cells other than tumor thyrocytes. This was also the case with the report by Paschke *et al.* (4) who sequenced subcloned TSHR DNA fragments and obtained only one and two clones with a disease-causing TSHR mutation from 33 and 96 clones examined, respectively. This is why we used RT-PCR for detection of TSHR mutation which should be present only in TSHR-expressing thyrocytes. The intensity of the mutant base signal was the same as that of the wild-type base signal (Fig. 1, left panel).

Our results do not exclude the possibility that the frequency of TSHR mutation in AFTNs in Japanese patients is less than that in European populations. Further extensive studies are necessary to determine the true prevalence of TSHR activating mutations in Japanese AFTNs, although it would be impossible to re-examine the samples previously defined as negative (15).

Leu512 is located in the third transmembrane domain consisting of amino acid residues 495–517. Amino acid sequences of residues 497–517 of the TSHR from all species and corresponding residues of the lutropin/choriogonadotropin receptor from all species are identical, suggesting the structural and/or functional importance of this domain of the TSHR.

In addition to the constitutive activation of the cAMP signal, L512R showed no increase in cAMP or IP by

TSH stimulation, which was observed with two constitutively activating deletion mutants Del(658–661) (7) and Del(613–621) (8). The very low surface expression (1/5 or less) of the mutant receptors may be responsible in Del(613–621) and Del(658–661) (7, 8, 30). However, L512R retained 52% $B_{\max}$ which was similar to or higher than that of the WT(5) transfectant (Table 1) that showed a good response to TSH (Fig. 2A). It is unlikely that the L512R mutant receptor is already maximally stimulated because the WT(5) transfectant showed higher cAMP and IP responses to high doses of TSH than L512R. L512R may have a conformation which is inaccessible to agonist or which prevents adoption of the maximally activated structure.

Three activating TSHR mutations involving the third transmembrane helix have been reported to date: S505R/N, V509A and L512R. As these three amino acid residues are spaced 3 or 4 amino acids apart and this portion is very likely to form a simple α -helix with a 3.6 amino acid turn, the three residues 505, 509 and 512 seem to face the same direction. This is in contrast to the sixth transmembrane helix where activating mutations are located consecutively (7). To determine whether identification of the three mutated amino acids in the third transmembrane helix was coincidental or was significant, we made a series of point mutations involving one of the residues 505–513 by substitution with Ser, Ala or Arg. No mutants involving residues other than 505, 509 or 512 showed constitutive activation of the cAMP signal (Fig. 3). Although we examined only one substitution mutant for each residue, it is likely that the three amino acid residues have a structurally specific role(s) in maintaining the inactive conformation of the TSHR. The evidence that such amino acid residues in the third transmembrane helix are located on the same surface of the α -helix suggests that one surface of the third transmembrane helix may have interhelical bonds with another helix to maintain the inactive conformation. Results obtained with rhodopsin (31), indicated that the third and seventh transmembrane helices play key roles in activation of the receptor by inducing a conformational change that results in displacement of transmembrane helices 3 and 7. This is in agreement with constitutive activation of the TSHR by these and several other point mutations in the third and seventh transmembrane domains of the TSHR.

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