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Clinical Significance of Heterozygous Carriers Associated with Compensated Hypothyroidism in R450H, a Common Inactivating Mutation of the Thyrotropin Receptor Gene in Japanese

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Loss-of-function mutations in the thyrotropin receptor (TSHR) gene were described as a syndrome characterized by thyroid hyposensivity to biologically active TSH, ranging from euthyroid to severe hypothyroidism. In Japanese, a common mutation in the TSHR gene is R450H, which demonstrated moderately impaired receptor function. We studied six subjects of Japanese origin whose major abnormality was persistent hyperthyrotropinemia by genetic sequence analysis of the TSHR gene. Three subjects were homozygous for the R450H mutation, whereas the three remaining subjects were single heterozygous. Homozygous subjects displayed mild hypothyroidism confirmed by moderately elevated basal TSH levels and excessive TSH response to TRH administration. Heterozygous subjects also demonstrated fully or partially compensated hypothyroidism, but less severe than that of homozygous subjects. More frequent involvement of the R450H mutation in the TSHR gene in Japanese was identified. In addition, a good correlation between phenotype and genotype was demonstrated in respect to biochemical analysis and drug dosage. Our observations showed clinical significance of heterozygosity associated with compensated hypothyroidism in spite of only mildly impaired receptor function.

Key Words: Thyrotropin receptor gene; TSH resistance; heterozygous; congenital hypothyroidism; inactivating mutation.

Introduction

Congenital hypothyroidism occurs at a rate of 1 in approx 3500 births *(1–3)*; 85% of congenital hypothyroidism cases are due to defects in thyroid development leading to glandular dysgenesis *(4)*. Thyroid dysgenesis is associated with complete agenesis or ectopic or hypoplastic development of the gland. Most cases of thyroid dysgenesis are sporadic. However, up to 2% of patients with thyroid dysgenesis have a family history of this disorder, suggesting the existence of contributing genetic factors *(5)*.

The thyrotropin receptor (TSHR), which is present at the surface of the thyroid follicular cell, is a G protein–coupled receptor consisting of seven transmembrane spanning regions and a large extracellular domain that mediates the effects of TSH and is important for the development and function of the thyroid gland *(6)*. TSHR is also known to be involved in the final stages of thyroid organogenesis. The TSHR gene is located on chromosome 14q31 *(7)* and the extracellular domain of the receptor is encoded by nine exons, whereas the transmembrane and intracellular portions are encoded by exon 10 *(8)*. In common with other G protein–coupled receptors, both activating and inactivating mutations have been described for the TSHR gene *(9)*. Loss-of-function in TSHR causes TSH resistance due to variably reduced sensitivity to TSH. Since 1995, 33 different loss-of-function mutations of the TSHR gene have been reported *(10–23)*. It has been shown that TSHR gene mutations can cause a wide spectrum of thyroid abnormalities, ranging from severe hypoplasia to an almost normal sized and structured thyroid gland *(24,25)*. In addition, thyroid hormone levels range from severe hypothyroidism to within normal limits. In previous studies, TSH resistance associated with a TSHR mutation was described to follow a recessive pattern of inheritance, because most probands with large TSH elevations were linked to homozygous or compound heterozygous mutations. More recently, small TSH elevations were observed in the familial cases of partial TSH resistance associated with heterozygous inactivating mutations in the TSHR gene *(19,21,26)*. This fact suggests the existence of a dominant mode of inheritance.

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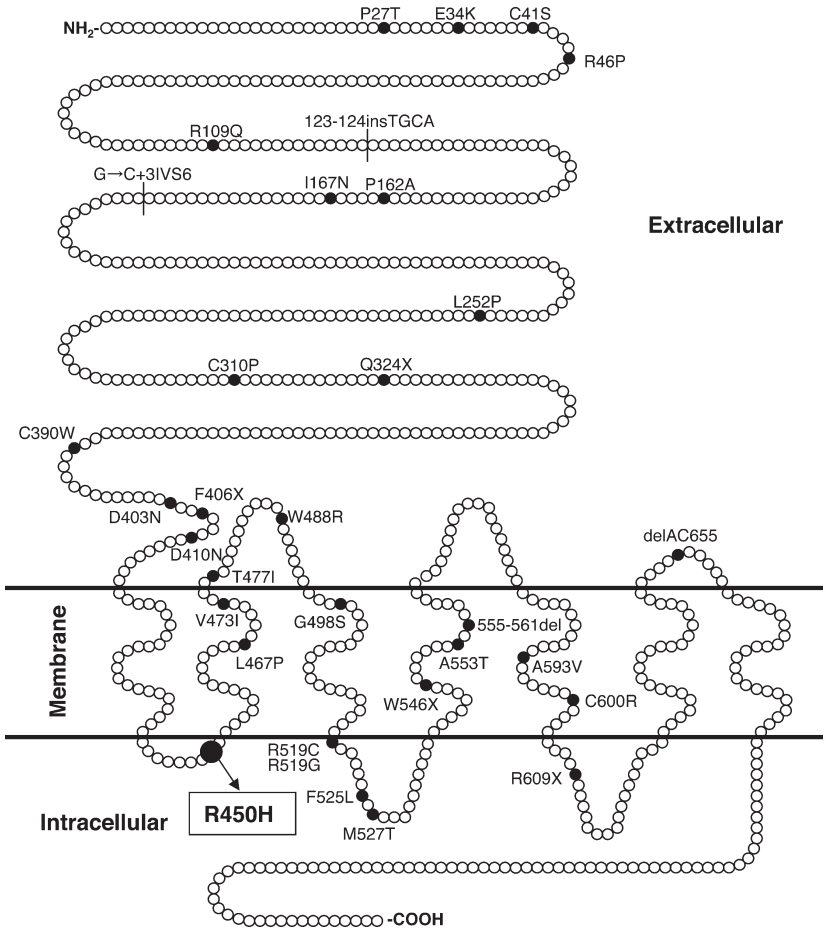

Fig. 1. Diagram of the thyrotropin receptor showing the positions of all the inactivating mutations reported to date. R450H is located in the first cytoplasmic loop.

In Japan, loss-of-function mutations in the TSHR gene have been previously reported in five cases *(18,23,27)*. In all of these cases, the individuals were found to harbor homozygous or compound heterozygous R450H mutations of the TSHR gene. In previous studies, it was reported that COS-7 cells transfected with the R450H mutation demonstrated moderately decreased TSH binding activity and a moderately decreased cell surface expression, in spite of normal intracellular synthesis *(18)*. Inactivating mutations in the TSHR gene occur in families of different ethnic backgrounds and geographical areas. The incidence suggests that the R450H TSHR mutation is a major contributor to congenital hypothyroidism or persistent hyperthyrotropinemia in the Japanese population.

For this study, we identified six patients of Japanese origin who harbored the R450H mutation in at least one allele. Three patients were homozygous for R450H, whereas the three remaining patients carried the R450H mutation in the heterozygous state. Heterozygous carriers were revealed to have fully or partially compensated hypothyroidism. We examined clinical and biochemical features among the affected subjects in detail and analyzed the correlation between phenotype and genotype.

Results

Genetic Analysis of the TSHR Gene

The sequence of the TSHR gene revealed that patients 1, 2, and 3 were homozygous for a G-to-A nucleotide substitution of His (CAC) in place of Arg (CGC) at codon 450 (R450H) located in the first cytoplasmic domain (Fig. 1). On the other hand, patients 4, 5, and 6 showed were only heterozygous for R450H (Table 1). The entire coding region of the TSHR gene was thoroughly sequenced to high accuracy in all of the subjects and no other sequence change was found except for a previously described receptor polymorphism *(28,29)*.

Clinical and Biochemical Observations

The biochemical and imaging characteristics are shown in Tables 1, 2, and 3. All subjects that were homozygous for R450H could be correctly diagnosed based on neonatal TSH screening, with measured TSH values on the dry blood spot between 18.2 and 49 μU/mL. On the other hand, among the subjects who were simply heterozygous for R450H, only patient 4 was referred because of hyperthyrotropinemia diagnosed on neonatal TSH screening; the TSH value of patient

Table 1
Results of TSHR Gene Sequence
and Detailed Biochemical Analyses of Thyroid Function for the Six Subjects

Patient	Genotype of TSHR gene	Basal TSH level (μU/mL)	TSH peak after TRH (μU/mL)	Thyroglobulin (ng/mL)
1	**R450H/R450H**	**6.3–54.8**	**59.1**	≦5 (18 yr)
2	**R450H/R450H**	**15.5–38.5**	Not available	24 (15 yr)
3	**R450H/R450H**	**15.9–26.0**	**132.6**	8 (9 yr)
4	**R450H**/wt	2.6–**11.9**	**36.4**	25 (14 yr)
5	**R450H**/wt	3.8–**9.0**	27.5	28 (4 yr)
6	**R450H**/wt	3.1–**7.5**	**45.9**	20 (14 yr)

Normal range value of serum TSH is 0.54–4.26 μU/mL, thyroglobulin is under 30 ng/mL, and the time of measurement is reported between parentheses. Peak TSH values above 35 μU/mL were considered hyperresponsive *(30)*. Antithyroid autoantibodies were negative in all these subjects. Abnormal data are reported in **bold**.

Table 2
Results of Imaging Studies and Perchlorate Discharge Test

Patient	Thyroid ultrasound	^{123}I scintigraphy	Perchlorate discharge test
1	Normal	Normal	Not available
2	Normal	Normal	Not available
3	Normal	Normal	Normal
4	Normal	Normal	Normal
5	**Slight hypoplasia**	Normal	Not available
6	Normal	Normal	Normal

In perchlorate discharge test, we diagnosed positive discharge when over 20% iodine released.

Table 3
Clinical and Biochemical Data of the Six Patients at the First Presentation at Our Laboratory

Patient	Sex	Age at first examination	TSH on dry blood spot (μU/mL)	Serum TSH (μU/mL)	fT_4 (ng/mL)	fT_3 (pg/dL)
1	M	33 d	**18.2**	**20.8**	1.2	4.4
2	M	27 d	**35.2**	**38.5**	1.2	4.6
3	F	17 d	**49**	**26.0**	1.3	5.3
4	F	31 d	**13.3**	**6.1**	1.5	5.8
5	F	58 d	Negative	**4.7**	1.7	3.6
6	M	6 yr	Negative	**7.4**	1.1	3.7

Normal range value of serum TSH is 0.54–4.26 μU/mL, fT_4 is 0.71–1.52 ng/mL, fT_3 is 2.39–4.06 pg/dL, respectively. fT_4 and fT_3 were considered to be within normal limits for their age. Patients 3 and 6 are sporadic. Patients 1 and 2 have the same parents; patients 4 and 5 also have the same parents, both parents for all four patients being of Japanese origin. Abnormal data are reported in **bold**.

4 was 13.3 μU/mL. Patients 5 and 6 did not show any abnormality on neonatal TSH screening.

At the first medical examination in our laboratory, serum TSH concentrations of subjects homozygous for R450H were 20.8–38.5 μU/mL, whereas those who were heterozygous were 4.7–7.4 μU/mL. In spite of slight to moderate serum TSH elevations, serum thyroid hormone levels were within normal limits. None showed any presence of thyroid antibody. Thyroglobulin levels showed a wide spectrum that ranged from a slight elevation above normal to a low, but still measurable, value. The TSH response to TRH administration was exaggerated in patients 1, 3, 4, and 6, whereas the high normal response was demonstrated in patient 5. The TSH peak after TRH stimulation was 59.1–132.6 μU/mL in the homozygous subjects and 27.5–45.9 μU/mL in the heterozygous subjects. Ultrasound scanning and ^{123}I iso-

Table 4
Progress of Replacement Therapy
with Levothyroxine and Clinical Assessment of Development

Patient	Start time at therapy	Maintenance dose (μg/kg/d)	DQ/IQ
1	20 mo	2.3–4.8	129/115
2	4 mo	2.4–5.1	129/121
3	17 d	3.9–10.1	104/115
4	2 yr	1.1–3.1	102/96
5	6 mo*	1.5–2.2	121/Not available
6	He did not undergo the therapy.		Not available

Maintenance dose represents the dose to keep serum TSH level within normal limits.

*The therapy was suspended at the age of 2 yr.

tope scanning revealed slightly small to normal sized thyroid glands in the normal position in all subjects. After administration of potassium perchlorate, no significant release of iodine was observed for patients 3, 4, and 6.

Because of prolonged moderate hyperthyrotropinemia or excessive TSH response to TRH stimulation, treatment with levothyroxine for all R450H homozygous subjects began within 2 yr after birth. Although they were considered to be treated with appropriate replacement doses of levothyroxine, serum TSH levels fluctuated between normal limits and slight elevation. Consequently, these patients needed frequent adjustment of their levothyroxine dosages in follow-up visits. Minimal doses of levothyroxine for euthyroid were 2.3–3.9 μg/kg/d. Subjects who were heterozygous for R450H did not necessarily need levothyroxine treatment. Levothyroxine replacement treatment for hyperthyrotropinemia was initiated in patient 4 at 2 yr of age. Patient 5 was temporarily treated with levothyroxine, whereas patient 6 underwent no treatment. During replacement therapy, thyroid functions of patients 4 and 5 maintained euthyroid without frequent adjustment of levothyroxide dosage. Their minimal doses of levothyroxine for euthyroid were 1.1–1.5 μg/kg/d. However, basal TSH levels in the case of no treatment were slightly elevated, 7.5–11.9 μU/mL. So far, clinical assessments including intelligence tests of the all affected patients revealed normal growth and development (Table 4).

Discussion

Five different subjects with inactivating TSHR mutations implicated in TSH resistance have been described previously in Japan *(18,23,27)*. Interestingly, all of the affected patients had an R450H mutation, either in the homozygous or compound heterozygous state. Our six subjects (four families) also possessed the R450H mutation. These facts suggest the possibility of frequent involvement of the R450H mutation of the TSHR gene in subjects with hyperthyrotropinemia in the local Japanese population.

When we divided the subjects into homozygous and heterozygous groups, we found that clinical features and imaging studies did not reveal a clear distinction. The degree of hyperthyrotropinemia, the peak values of TSH after TRH administration, and the dose of levothyroxine required to maintain euthyroid state clearly differed significantly between the two groups. For all of the homozygous subjects, we found it impossible to keep their basal TSH level within normal limits without levothyroxine replacement therapy. Our results also demonstrated that those subjects who were heterozygous for R450H might have exhibited a biochemical picture consistent with compensated hypothyroidism.

In previous studies of impaired receptor functional analysis detected in Japanese, R450H showed moderately decreased TSH binding and moderately decreased cAMP response to TSH *(18,23)*. A close relationship was additionally reported between serum TSH levels and functions of the mutant TSH receptors. Although the degree of receptor impairment of R450H may be mild among the loss-of-function mutations in TSHR, our observation of R450H heterozygous subjects exhibited the phenotype of mild or subclinical hypothyroidism. One of the three subjects heterozygous for the R450H mutation has continued the levothyroxine replacement treatment.

Among heterozygous carriers of inactivating TSHR mutations, persistent hyperthyrotropinemia, excessive TSH response to TRH stimulation, and a slightly hypoplastic thyroid gland suggest the existence of mild congenital hypothyroidism. However, in the previous studies, almost all subjects harboring a simple heterozygous mutation in the TSHR gene were consanguinity of propositus and had not noted any thyroid dysfunction until their analysis. Therefore, only a small number of the single heterozygous patients were followed up as hyperthyrotropinemia or mild hypothyroidism patients, and detailed thyroid function analyses for the heterozygous carriers of loss-of-function TSHR gene mutations have only occasionally been reported *(19,21,26)*. On the other hand, some heterozygous carriers who clinically and biochemically demonstrated euthyroid showed a normal TSH response to TRH stimulation *(14,19)*. Various factors such as the degree of impairment of TSHR function or the change of thyroid hormone needs with aging have affected heterozygous carriers of TSHR gene mutations may help explain why they showed various phenotypes from euthyroid to mild hypothyroidism.

Unfortunately, it may be difficult to sustainably carry out longitudinal follow-up for such subclinical or mild hypothyroidism patients. Furthermore, opinion is divided as to whether or not replacement therapy is necessary with respect to improving the quality of life of subclinical patients. However, it is impossible to deny that patients with subclinical hypothyroidism are at considerable risk for adverse neurodevelopmental consequences. No guideline exists for such subclinical patients but the presence of excessive TSH response to TRH administration strongly indicates that they

should be diagnosed with hypothyroidism and accordingly receive replacement therapy *(30)*. The authors of a recent study advocate the administration of low-dose levothyroxine to infants with borderline hypothyroidism in order to prevent possible adverse effects of mild hypothyroidism on the developing brain *(31)*. Furthermore, if left untreated, these affected patients may develop overt hypothyroidism during periods such as puberty and pregnancy, when a much higher amount of thyroid hormone is needed *(32,33)*. Therefore, clinical and biochemical longitudinal follow-up plays a crucial role in monitoring the health of heterozygous carriers of TSHR gene mutations like R450H.

In conclusion, we studied six Japanese subjects with TSH resistance who were homozygous or heterozygous for the R450H mutation of the TSH receptor. Our results suggest that the R450H mutation may be majorly associated with subclinical or mild hypothyroidism in Japanese patients having a normally positioned thyroid gland. We observed a good correlation between phenotype and genotype among the subjects with R450H TSHR defects in Japan. In addition, subjects who were heterozygous for R450H demonstrated persistent moderate hyperthyrotropinemia and high normal to excessive TSH response to TRH administration. We emphasize the existence of fully or partially compensated hypothyroidism in the heterozygous carriers even if the degree of their receptor impairment is mild.

Materials and Methods

Subjects

We studied six subjects with neonatal to juvenile evidence of hyperthyrotropinemia. Patients 1 and 2 have the same parents, and patients 4 and 5 also have the same parents. All the patients were born to nonconsanguineous parents of Japanese origin, following an unremarkable pregnancy and spontaneous delivery at full term. Result of neonatal TSH screening on dry blood spot were above the normal range in four of six cases. In the two remaining cases, hyperthyrotropinemia was diagnosed after chance TSH screening, in patient 5 at 2 mo old because of an older sister's history and in patient 6 after a complaint of short status at 6 yr old for the first time (Table 3).

The five subjects who were diagnosed with hyperthyrotropinemia as infants had ossification centers of the knee and suggested that apparent hypothyroidism was not present without persistent jaundice. Although patient 6 complained of short status, at the time of presentation the 6-yr-old boy was 106.8 cm (−1.40 SD), and his physical and intellectual development were normal. No subject had a goiter or otherwise appeared physically abnormal. We followed them up for hyperthyrotropinemia.

Biological and Radiological Examinations

Basal serum TSH, fT_4, fT_3, and thyroglobulin were continuously measured in each subject. Thyroid antibodies were also measured to distinguish autoimmune hypothyroidism. TRH stimulation tests were performed in all subjects except for patient 2. The effect of 10 μg/kg of TRH was quantified by measuring the concentration of TSH in blood samples at 0, 30, 60, 90, and 120 min after TRH administration.

Ultrasound scanning of the thyroid gland and neck was undertaken. ^{123}I isotope scanning was performed in all subjects and a perchlorate discharge test was also administered in patients 3, 4, and 6. In a perchlorate discharge test, the release of accumulated iodine was measured at least every 1 h for a maximum of 2 h after administration of potassium perchrolate.

Amplification of the TSHR Gene and Sequence Analysis

Molecular investigation of the TSHR gene was performed under the approval of the institutional review board of the Nagoya City University Graduate School of Medical Sciences' Institute for Molecular Research, and informed consent was obtained from all patients' families to participate in the TSHR gene studies.

Genomic DNA was isolated from 200 μL of peripheral blood lymphocytes using the QIAamp DNA Blood Mini Kit (QIAGEN, Tokyo, Japan). The entire TSHR gene of each subject was sequenced using oligonucleoside primers on the basis of the published sequence of the human TSHR gene *(26,34)*.

PCR amplification was performed with 1 μL samples of each DNA preparation in a 20 μL reaction volume containing 0.5 U gold Taq polymerase (Takara, Otsu), 125 μ*M* of each deoxyribonucleoside triphosphate, 0.5 μL of each primer pair, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, and 10 m*M* Tris-HCl (pH 8.3). PCR was initiated by extended denaturation (5 min at 95°C), followed by 40 cycles of denaturation (1 min at 94°C), annealing (1 min at 57°C), and extension (1 min at 72°C) in a programmable heat-block system (GeneAmp PCR System 9700; Applied Biosystems, Chiba, Japan). The amplified DNA products were separated by electrophoresis through a 2% agarose gel stained with ethidium bromide, and visualized using an ultraviolet transilluminator. The desired amplified products were extracted from the agarose gel and purified using a commercial kit (QIAquick gel extraction kit; QIAGEN) and were directly sequenced in both directions using a fluorescent dye terminator cycle system with the specific primers described above and a commercial DNA sequencing kit (Applied Biosystems) and autoanalyzer (ABI PRISM 310 Genetic Analyzer; Applied Biosystems), according to the manufacturer's instructions.

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