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Original Article

Identification of compound heterozygous *TSHR* mutations (R109Q and R450H) in a patient with nonclassic TSH resistance and functional characterization of the mutant receptors

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Abstract. Genetic defects of the TSH receptor (TSHR) signaling pathway cause a form of congenital hypothyroidism (CH) known as TSH resistance. Consistent with the physiological understanding that thyroidal iodine uptake is up-regulated by TSHR signaling, most patients with TSH resistance have low to normal thyroidal ^{123}I uptake representing the classic TSH resistance. However, paradoxically high ^{123}I uptake was reported in four molecularly-confirmed patients indicating nonclassic TSH resistance. Here, we report the fifth patient with the nonclassic phenotype. He was a 12-yr-old CH patient and treated with levothyroxine. At the age 11 yr, he showed slightly small thyroid gland and elevated thyroidal ^{123}I uptake. Genetic analysis showed that he was compound heterozygous for two known missense mutations (Arg109Gln and Arg450His) in the TSHR gene. Further, the signal transduction of Arg109Gln-TSHR was defective in both Gs- and Gq-coupled pathways, while Arg450His-TSHR showed Gq-dominant defect. ^{123}I uptake was evaluated earlier in 16 patients with TSH resistance, and a correlation between TSH levels and ^{123}I uptake was shown in patients with specific genotypes (Arg450His or Leu653Val). Collectively, we have re-confirmed that the emergence of the nonclassic phenotype requires two factors: mutant TSHR with Gq-dominant coupling defect and relatively high levels of serum TSH.

Key words: congenital hypothyroidism, TSH resistance, TSH receptor, mutation, genetics

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Introduction

Congenital hypothyroidism (CH), which occurs in 1 in 3,000 to 4,000 births, is the most common congenital endocrine disorder (1). CH causes, when left untreated, irreversible growth retardation and intellectual disability. Importantly, these sequelae can be prevented by

early diagnosis and initiation of levothyroxine replacement therapy. Developed countries have introduced newborn screening for CH wherein the disease is screened by measuring blood-spot TSH (or T_4) on filter papers. This screening now enables pediatricians to diagnose a vast majority of CH patients and to start treatment early.

CH is a heterogeneous disorder characterized by inadequate production of thyroid hormones from the thyroid gland. Based on the morphology of the gland, CH cases can be clinically classified into three major categories. The first category is thyroid dysgenesis, which includes thyroid hypoplasia, aplasia, and ectopia, where the thyroid gland cannot produce a sufficient amount of hormones because of insufficient thyroid follicular cells. The second category includes inborn errors of thyroid hormone biosynthesis, collectively known as thyroid dyshormonogenesis. Patients with thyroid dyshormonogenesis present goiter, which results from hyper-stimulation of the gland by elevated TSH. The third category represents CH with morphologically normal thyroid gland. This heterogeneous category includes mild forms of thyroid dysgenesis and thyroid dyshormonogenesis.

Stimulation of the TSH receptor (TSHR) by TSH is an indispensable initial step of ligand-dependent thyroid hormone production. Thus, mutations in the TSHR gene (*TSHR*) cause CH that is known as TSH resistance. Classically, clinical pictures of TSH resistance were thought to be determined by the residual activity of the mutant receptors. In this simplistic model, patients with two nonfunctional *TSHR* alleles would have thyroid hypoplasia and low thyroidal ^{123}I uptake, whereas patients having at least one allele with residual activity would have normal-sized gland with normal ^{123}I uptake (2). However, an atypical form of TSH resistance (*i.e.,* nonclassic TSH resistance), which is characterized by paradoxical elevation of ^{123}I uptake, has also been reported (3, 4). The atypical phenotype is related to specific *TSHR* mutations (p.Arg450His and p.Leu653Val), and is explained by the characteristic signaling properties of the two mutant receptors (Gq-dominant coupling defect) (3, 4). Only four *TSHR* mutation-carrying patients with the nonclassic phenotype have been reported to date. In this report, we describe the fifth patient with the nonclassic phenotype and compound heterozygous *TSHR* mutations.

Patient and Methods

Patient

The patient, a 12-yr-old Japanese male, had a high blood-spot TSH level (39 mU/L; cut-off level 10 mU/L) on newborn screening for CH, and was referred to us at the age of 5 d. Blood tests revealed a high serum TSH level (13 mU/L; reference 1.7–9.1) and a normal free T_4 level (1.2 ng/dL; reference 0.9–2.3). Due to normal free T_4 level and lack of CH-related symptoms, the patient was carefully followed-up without treatment. He showed seemingly normal growth and development; however, his serum free T_4 levels decreased gradually. At the age of 11 mo, levothyroxine replacement therapy was started at 20 μg/d because the serum TSH level exceeded 10 mU/L (Fig. 1). At the age of 11 yr, we re-evaluated his thyroid function with stopping treatment (levothyroxine, 100 μg/d) for 4 wk. He had a high serum TSH level (68 mU/L) with normal thyroid hormone levels (free T_4 1.1 ng/dL, free T_3 3.1 pg/mL) and a slightly high serum thyroglobulin level (61 ng/mL; reference < 30). Ultrasonography demonstrated slightly small thyroid gland (–1.4 SD) (5). ^{123}I uptake was high (60.2% at 24 h; reference 8–40), but perchlorate test was negative (discharge rate 5%; reference < 10). He was diagnosed as having permanent CH, and levothyroxine replacement therapy was restarted.

Mutation detection

This study was approved by the Ethics Committee of Keio University School of Medicine. A written informed consent for the molecular study was obtained from the parents

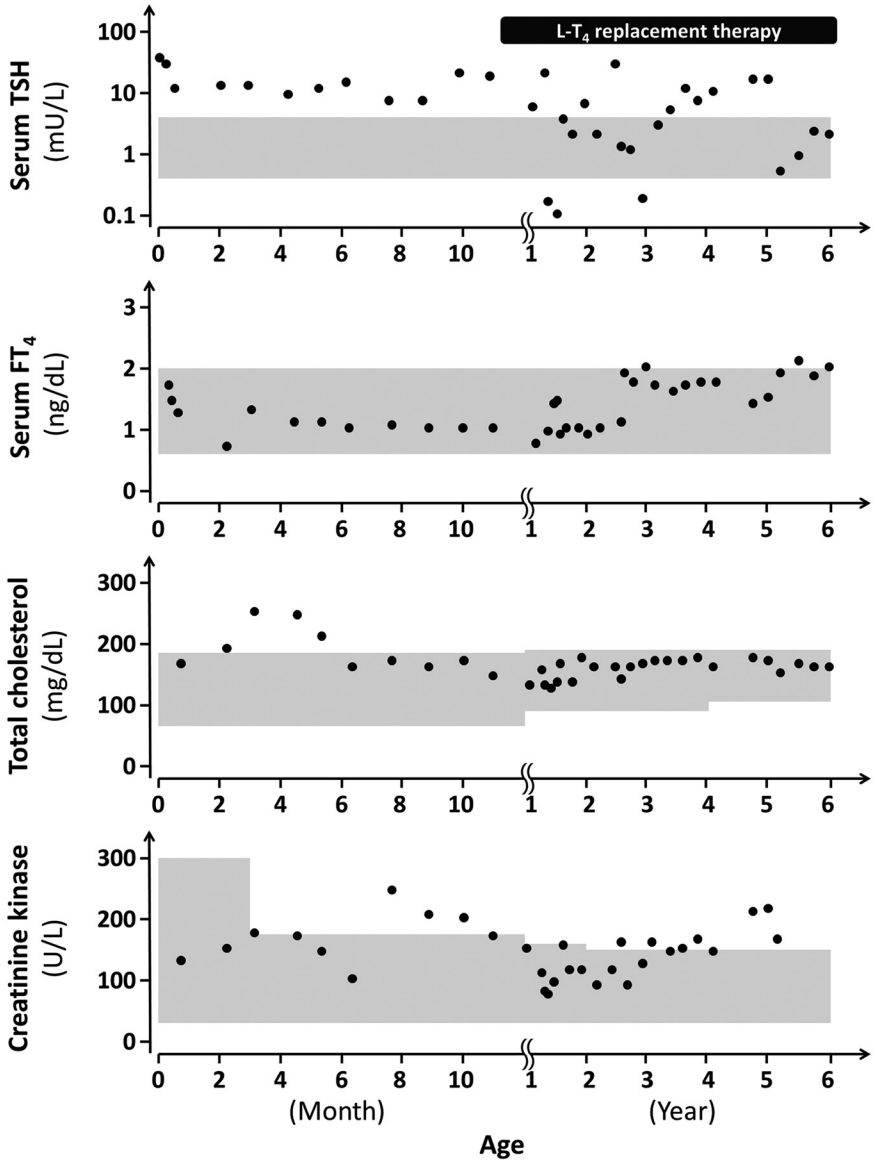

Fig. 1. Levels of serum TSH, free T_4, total cholesterol, and creatinine kinase in the patient are shown. Gray areas indicate age-specific normal ranges. The patient was treated with L-T4 from the age of 11 mo.

of the patient. Genomic DNA samples were collected from the patient, his sister, and his parents. Eleven known genes associated with CH (*DUOX2, DUOXA2, FOXE1, IYD, NKX2-1, PAX8, SLC5A5, SLC26A4, TG, TPO,* and *TSHR*) were analyzed with the use of a next-generation sequencer MiSeq (Illumina Inc., San Diego, CA, USA) according to the SureSelect protocol (Agilent Technologies, Santa Clara, CA, USA) as described previously (6). Base calling, read filtering, and demultiplexing were performed with the standard Illumina processing pipeline. We used BWA 0.7.5 (7) for alignment against the human reference genome (NCBI build 37; hg19) with the default settings. Local realignment, quality score recalibration, and variant calling were performed by GATK 3.2.0 (8) with the default settings. We used ANNOVAR (9) for annotation of called variants. The detected mutation was confirmed by standard PCR-based

Sanger sequencing using previously described methods (10).

Functional characterization of mutant TSHR

The N-terminal HA-tagged wild type (WT)-TSHR has been described previously (4). The p.Arg109Gln and p.Arg450His mutations were introduced by site-directed mutagenesis (PrimeSTAR Mutagenesis Basal Kit, TAKARA Bio Inc., Otsu, Japan) according to the manufacturer's instructions. HEK293 cells were maintained in DMEM supplemented with 50 U/mL penicillin, 50 μg/mL streptomycin, and 10% fetal bovine serum. For transient transfection, we used Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol.

We performed the luciferase assay to quantify TSH-stimulated activation of the Gs/cAMP and Gq/Ca^{2+} pathways. To assess the activation of the Gs/cAMP pathway, we seeded HEK293 cells into a 96-well plate at about 90% confluence, and transfected them with 10 ng each TSHR construct (empty vector, WT construct, and constructs containing p.Arg109Gln or p.Arg450His) along with 50 ng of the reporter vector (CRE-luc; pGL4.29, Promega, Madison, WI, USA). Forty-eight hours after transfection, the medium was removed, and cells were incubated with 100 U/L of bovine TSH in DMEM for 3 hours at 37°C. Luciferase activity was measured using One-Glo Luciferase Assay System (Promega). Further, to assess the activation of the Gq/Ca^{2+} pathway, we established a stable HEK293 cell line with NFATRE-luc sequence derived from pGL4.30 (Promega) in the genome using a piggybac technique (System Biosciences, Palo Alto, CA, USA). These cells were seeded into a 24-well plate at about 70% confluence and transfected with 600 ng each TSHR construct. Twenty-four hours after transfection, cells were harvested and resuspended in DMEM containing 10 ng/mL phorbol 12-myristate 13-acetate. The transfected cells were reseeded into a 96-well plate about 90% confluence. Forty-eight hours after transfection, the medium was removed, and cells were incubated with 100 U/L of bovine TSH in DMEM for 3 h at 37°C. We added 1 μM Ionomycin (Sigma) when we measured NFATRE-luc activities. Luciferase activity was measured using the Nano-Glo Luciferase Assay System (Promega).

The activity of each mutant is expressed as percentage (mean ± SEM) of WT activity. The background activity, which was measured with the use of mock-transfected cells, was set to 0%. Experiments were conducted in triplicate and repeated at least thrice.

Literature review

A review of the articles published prior to July 1, 2017 was conducted independently by two authors (C.S. and S.N.). PubMed and Google Scholar search engines were used to identify relevant articles using keywords, such as "TSHR", "mutation", "TSH resistance", and "thyrotropin resistance". Serum TSH levels and ^{123}I uptake values of biallelic *TSHR* mutation carriers were obtained and analyzed.

Results

Mutation detection

Next-generation sequencing-based comprehensive genetic screen of the patient let us to identify two previously reported *TSHR* mutations: c.326G>A, p.Arg109Gln (11) and c.1349G>A, p.Arg450His (12). No mutation was found in the other sequenced genes. The presence of the mutations was confirmed by Sanger sequencing (Fig. 2A). Familial analysis showed that the mutations p.Arg109Gln and p.Arg450His were inherited from the father and mother, respectively. Unaffected sister did not have either of these mutations (Fig. 2B).

Functional characterization of the mutant TSHR

Previously, we have shown that p.Arg450His-

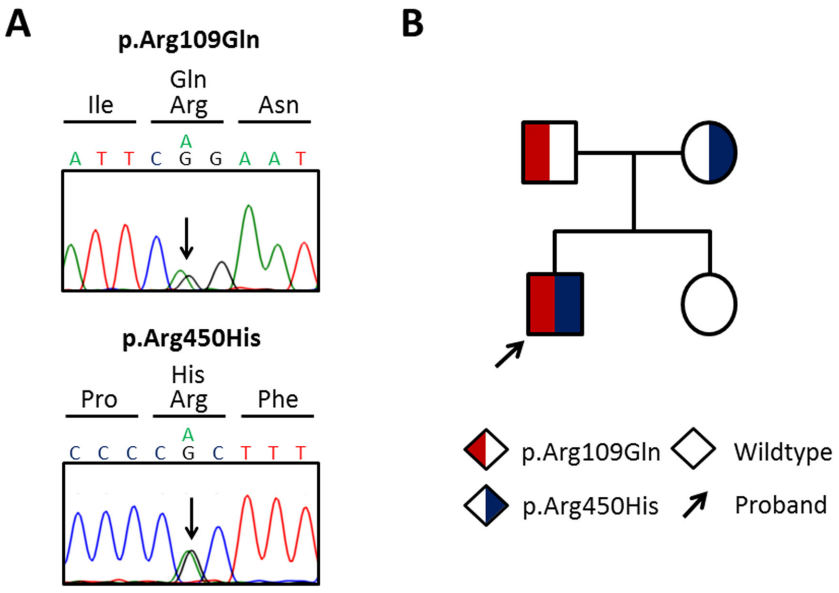

Fig. 2. Identification of compound heterozygous *TSHR* mutations p.Arg109Gln and p.Arg450His. (A) Partial electropherograms of the PCR products of *TSHR*. The heterozygous substitutions of glutamine in place of Arg109 and histidine in place of Arg450 are indicated by arrows. (B) Pedigree of the patient is shown. The parents of the proband had the mutation in heterozygous state (father: p.Arg109Gln; mother: p.Arg450His). His sister did not have either of the mutations.

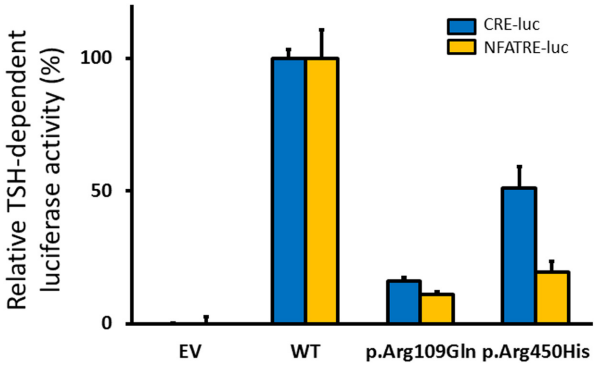

Fig. 3. TSH-dependent activation of Gs- and Gq-coupled signaling of the p.Arg109Gln and p.Arg450His mutations. HEK293 cells were transfected with each TSHR-expressing vector [wild type (WT), p.Arg109Gln, or p.Arg450His] or empty vector (EV), and were incubated with or without 100 U/L of bovine TSH. Next, we measured luciferase activity. Note that the p.Arg450His mutation displayed a profound defect in Gq-coupled signaling, whereas p.Arg109Gln showed "balanced" defects in both Gs- and Gq-coupled signaling.

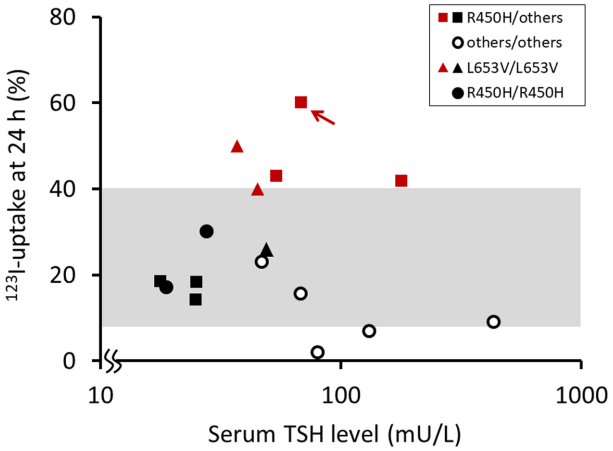

Fig. 4. A scatter plot of radioiodine (^{123}I) uptake values and serum TSH level of biallelic *TSHR* mutation carriers is shown. Filled symbols represent patients having at least one allele with Gq-dominant coupling defect, whereas squares, triangles, and circles represent homozygous Arg450His, homozygous Leu653Val, and compound heterozygous with Arg450His and others, respectively. Red symbols represent patients with nonclassic TSH resistance, and black ones indicate those with classic TSH resistance. The patient described in the present study is indicated by the arrow. White circles represent patients with TSH resistance but without Gq-dominant coupling defect. In patients carrying at least one allele with Gq-dominant coupling defect, a correlation between serum TSH levels and ^{123}I uptake values was seen.

TSHR is characterized by partial loss of Gs/cAMP signaling and profound loss of Gq/Ca^{2+} signaling (*i.e.,* Gq-dominant coupling defect) *in vitro* (4). However, the p.Arg109Gln mutation has been evaluated only for the Gs/cAMP pathway (11). Thus, it remained unknown whether the p.Arg109Gln mutation has the Gq-dominant coupling defect. Therefore, we evaluated the effect of the p.Arg109Gln mutation on the Gs- and Gq-coupled signaling pathways in the present study. When cells transfected with each TSHR vector (empty vector, WT-TSHR construct, and mutant constructs— Arg109Gln-TSHR or Arg450His-TSHR) were stimulated by 100 U/L bovine TSH, TSH-dependent CRE-luc activation (*i.e.,* Gs/cAMP pathway reporter) was 16.1 ± 1.2% (mean ± SEM) for Arg109Gln-TSHR and 51.1 ± 8.1% for Arg450His-TSHR as compared with that in the case of WT-TSHR (Fig. 3). Similarly, we evaluated TSH-dependent NFATRE-luc activation (*i.e.,* Gq/Ca^{2+} pathway reporter), and showed that the relative activities were 10.9 ± 1.2% for Arg109Gln-TSHR and 19.4 ± 0.2% for Arg450His-TSHR as compared with that in the case of WT-TSHR (Fig. 3). From these findings, we re-confirmed that the p.Arg450His mutation caused the Gq-dominant coupling defect, while the p.Arg109Gln mutation caused "balanced" Gs- and Gq-coupling defects.

Correlation between serum TSH levels and ^{123}I uptake in biallelic TSHR mutation carriers

To understand the relationship among *TSHR* genotypes, serum TSH levels, and thyroidal ^{123}I uptake, we collected relevant clinical details from the literature and our unpublished data, and drew a scatter plot (Fig. 4) (3, 10, 12–16). There were significant log-linear correlations (P values < 0.05) between serum TSH levels and thyroidal ^{123}I uptake among patients having at least one allele with Gq-dominant coupling defect (p.Arg450His or p.Leu653Val) while such correlation was not observed among those without the alleles. Among patients having at least one allele with the Gq-dominant coupling defect, serum TSH levels were significantly higher in patients with high ^{123}I uptake (median, 53.8 mU/L) than in those with normal ^{123}I uptake (median, 25.0 mU/L) (P < 0.05 by Mann-Whitney U test).

Discussion

In the present report, we describe a CH patient with compound heterozygous *TSHR* mutations (Arg109Gln and Arg450His). The patient demonstrated paradoxically increased ^{123}I uptake, which was compatible with the nonclassic TSH resistance. In our previous study (4), we proposed that *TSHR* mutations with Gq-dominant coupling defect (*e.g.,* p.Arg450His and p.Leu653Val) would cause the paradoxical phenotype. We therefore examined whether p.Arg109Gln gave rise to the Gq-dominant defect, and found that the mutation did not cause the defect. We presume that the Gq-dominant defect in the patient's thyroid gland was chiefly due to the opposite *TSHR* allele with the p.Arg450His mutation.

The study subject described in this study is the fifth molecularly-confirmed patient with nonclassic TSH resistance. *TSHR* genotypes found in previously reported patients include p.Leu653Val (homozygous) (3), p.Thr145Ile and p.Arg450His, and p.Arg450His and Ile611Asnfs*10 (4). All the patients had at least one allele with p.Arg450His or p.Leu653Val; these alleles have been proven to have the Gq-dominant coupling defect (3, 4). Having one allele with the Gq-dominant coupling defect does not always result in the nonclassic TSH resistance. As shown in Fig. 4, patients with p.Arg450His or p.Leu653Val can have normal range of ^{123}I uptake values if the degree of TSH elevation is modest. We speculate that relatively increased serum TSH level, in addition to mutant receptor(s) with Gq-dominant coupling defect, is required for the development of the nonclassic phenotype. Thyroidal iodine uptake is regulated by the Gs/cAMP pathway via controlling the protein expression level of sodium iodine symporter (NIS) (17). In patients carrying *TSHR* mutation(s) with

Gq-dominant coupling defect, overexpression of NIS and resultant elevation of ^{123}I uptake can occur only if TSH receptor is stimulated enough to exceed relatively mild coupling defect to Gs.

Previously reported patients with nonclassic TSH resistance had slightly small thyroid gland (4). Similar morphology of the thyroid gland was also seen in our patient. In general, size of the thyroid gland and its iodine uptake are correlated in CH. Patients with thyroid hypoplasia tend to have low ^{123}I uptake, whereas most patients with goiter (*i.e.,* thyroid dyshormonogenesis), except for NIS defect, have high ^{123}I uptake. Coexistence of marginally small thyroid gland and high ^{123}I uptake is quite unusual. Therefore, we presume that the imbalance between thyroid size and iodine uptake would be a clinical key to suspect nonclassic TSH resistance.

In conclusion, we described the fifth molecularly-confirmed *TSHR* mutation carrier (p.Arg109Gln and p.Arg450His) with nonclassic TSH resistance. We assessed the balance between the Gs- and Gq-coupling signaling pathways for the two mutant TSHR *in vitro*, and found that the p.Arg109Gln mutation did not have the Gq-dominant coupling defect. Our experience further strengthens the hypothesis that the development of the nonclassic TSH resistance phenotype requires two factors: at least one *TSHR* allele with the Gq-dominant coupling defect and a relatively high serum TSH level.

Conflict of Interest: The authors have no conflict of interest to disclose.

Acknowledgments

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