

Identification and functional characterization of a novel thyrotropin receptor mutation (V87L) in a Chinese woman with subclinical hypothyroidism

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Abstract. The thyrotropin receptor (TSHR) gene has been defined as a highly mutable gene. Mutations in the TSHR gene result in either gain or loss of the receptor function. Subclinical hypothyroidism (SH) is a clinical condition defined as an elevated serum TSH level associated with normal free thyroxine and free triiodothyronine. Chronic autoimmune thyroiditis is the most frequent cause of subclinical hypothyroidism in adults. In rare cases, a loss-of-function mutation of TSHR is the cause of SH. In the present study, a novel TSHR mutation (V87L; confirmed to be a loss-of-function mutation) was identified in a 59-year-old Chinese woman, as the potential cause of the patient's subclinical hypothyroidism. The case may provide valuable insight into the etiology of SH.

Introduction

The thyrotropin receptor (TSHR) is a G-protein-coupled receptor consisting of seven transmembrane-spanning regions and a large extracellular domain that mediates the effect of TSH in thyroid development, growth and hormone synthesis. TSH exerts its biological effects through binding with TSHR to activate different effectors, predominantly adenyl cyclase and phospholipase C (1). Previous molecular analyses have revealed that mutations in the TSHR gene result in various thyroid dysfunctions (2).

Subclinical hypothyroidism (SH) is a clinical condition defined as an elevated serum TSH level associated with

normal free thyroxine (FT4) and free triiodothyronine (FT3). The patient is usually asymptomatic; however, a thorough evaluation of patients has indicated consequences for quality of life, cognitive abilities, cholesterol metabolism, heart rate, bone mineral density and atherogenesis (3). Chronic autoimmune thyroiditis is the most frequent cause of subclinical hypothyroidism in adults (4), and its diagnostic hallmarks are circulating thyroglobulin antibody (TgAb), thyroid peroxidase antibody (TPOAb) or TSHR-blocking antibodies. A hypoechoic pattern of the thyroid gland at ultrasound examination is also observed in autoimmune thyroiditis (5). In rare cases, resistance to TSH is the cause of SH. Resistance to TSH is a syndrome in which the thyroid displays a variable degree of hyposensitivity to a biologically active TSH molecule (6). The condition may be a result of abnormalities in the TSHR (7-13). Loss-of-function (LOF) mutations of TSHR always cause hypothyroidism, with the severity ranging from SH to overt hypothyroidism depending on the degree of the mutation (14). The present study reported the case of a woman with SH with a TSHR mutation (V87L) who tested negative for circulating TgAb and TPOAb.

Patients and methods

Case report. A 59-year-old female patient with a 12-year history of goiter was admitted to the Department of General Surgery, Xin Hua Hospital affiliated to Shanghai Jiaotong University School of Medicine in May 2010 for thyroid surgery. The patient was not receiving any medication at the time and the primary complaint was of pain in the neck region. The present study was approved by the Ethics Committee of Xinhua Hospital Affiliated to Shanghai Jiaotong University School of Medicine. Written informed consent was obtained from the patient. Laboratory investigations revealed a total triiodothyronine value of 1.01 nmol/l (normal range, 0.92-2.79 nmol/l), total thyroxine value of 60.7 nmol/l (normal range, 58.1-140.6 nmol/l), FT3 value of 3.8 pmol/l (normal range, 3.5-6.5 pmol/l), FT4 value of 12.74 pmol/l (normal range, 11.5-22.7 pmol/l) and a TSH value of 10.01 mU/l (normal range, 0.55-4.78), indicating subclinical hypothyroidism. The anti-TPOAb and anti-TgAb assessments were negative.

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Thyroid ultrasound revealed a normal sized thyroid gland (right lobe, 3.8x2.0x1.1 cm; left lobe, 3.9x1.9x1.0 cm) with a cystic nodule (2x2.5 cm in diameter) in the left lobe. A ^{99m}Tc scintiscan revealed a cold nodule. The patient experienced internal bleeding in the cystic nodule after feeling a sudden pain in the neck, and was subjected to subtotal thyroidectomy one week after his admission. Histologically, the nodule was benign. Immediately after the thyroidectomy, a tissue sample was obtained from the normal tissue and was shock-frozen in liquid nitrogen.

Gene sequence analysis. DNA samples were extracted from the patients' thyroid tissue using a DNA extraction kit (Omega Bio-Tek, Inc., Norcross, GA, USA). TSHR gene-coding exons were sequenced by direct DNA sequencing (Sangon Biotech Co., Ltd., Shanghai, China).

Construction of expression vectors and site-directed mutagenesis. RNA samples were extracted from the patients' thyroid tissue using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and the full-length TSHR complementary DNA (cDNA) was synthesized using the RNA as the template. The primers used for reverse transcription were as follows: Sense, 5'-CTAAGAGGTACC GAGTCCCGTGGAAAATGA-3'; and antisense, 5'-CCT CTAGACGCCCACTTACAAAACCGTTTGC-3' (Sangon Biotech Co. Ltd.). The reverse transcription reaction mixture included a Takara Biotechnology Co., Ltd transcription kit (Dalian, China) and the total volume was 50 μl . The reaction process was performed at 25°C for 10 min, 37°C for 60 min, then 95°C for 5 min. The mutant TSHR cDNA fragment was subcloned into a pcDNA3.1 plasmid (Invitrogen; Thermo Fisher Scientific, Inc.). The mutant TSHR-pcDNA3.1 was used as a template for mutagenesis to wild-type TSHR plasmid TSHR-pcDNA3.1 using the Site-Directed Mutagenesis kit (Beijing SBS Genetech Co., Ltd., Beijing, China) according to the manufacturer's protocol. All final constructs were verified by direct DNA sequencing.

Cell culture and in vitro functional analysis. Mutant and wild-type plasmids were stably transfected into Chinese hamster ovary (CHO) cells (Chinese Academy of Sciences, Beijing, China) in Ham's F12 medium with 5% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) and appropriate antibiotics using Lipofectamine reagent enhanced with Plus Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Surviving cells were selected with 600 mg/l geneticin (G418; Sigma-Aldrich, St. Louis, MO, USA). A bulk of G418-resistant colonies was used for the subsequent assessments.

Immunoblot analysis. Crude cell extracts were prepared from the transfected cells using a protein extraction kit and concentration was assessed using a bicinchoninic acid assay kit (Beyotime Institute of Biotechnology, Haimen, China), both in accordance with the manufacturer's instructions. Following this, 30 μg protein per sample was separated by 10% SDS-PAGE. Proteins were transferred to a polyvinylidene fluoride membrane and blocked with 5% non-fat milk in TBST buffer for 2 h at room temperature with continuous

agitation. Membranes were then incubated with a monoclonal anti-TSHR antibody (cat. no. MA3-217; Thermo Fisher Scientific, Inc.) diluted 1:500 and anti-beta actin antibody (cat. no. A01010; Abbkine, Wuxi, China) diluted 1:5,000 overnight at 4°C. This was followed by washing and incubation with horseradish peroxidase-labeled rat anti-mouse immunoglobulin G secondary antibody (cat. no. IH-0011; Dingguo Changsheng Biotechnology Co. Ltd, Beijing, China) at a dilution of 1:10,000 in TBST buffer for 2 h at 37°C. The signal was analyzed using enhanced chemiluminescence detection (Merck Millipore, Darmstadt, Germany). ImageJ software v. 4.18 (imagej.nih.gov/ij/) was used to quantify the protein

Analysis of competitive TSH binding. Bovine TSH (bTSH; Sigma-Aldrich) radio-iodination was performed according to the chloramine-T method as previously described (4-11). The transfected cells were re-seeded onto 12-well plates (400,000 cells/well). After 48 h, the medium was removed and the cells were incubated with ^{125}I -TSH (1×10^5 counts/ml; Shanghai Radioimmunity Institute, Shanghai, China) alone and in the presence of various concentrations of unlabeled bTSH (0, 0.1, 1, 10, 25, 50, 100, 150, 200, 500 and 1,000 mIU/ml) in the binding buffer (final volume, 0.6 ml) for 1.5 h at 37°C. Subsequently, the buffer was removed, the cells were lysed with 0.6 ml of 1 M NaOH and the radioactivity was quantified by a gamma-counter (Multi-Crystal Gamma Counter LB 2111, Berthold Technologies, Bad Wildbad, Germany) to determine TSH binding (B).

Analysis of TSH-stimulated cyclic adenosine monophosphate (cAMP) production. The cells were reseeded onto 96-well plates (25,000 cells/well). After 48 h, the medium was removed and the cells were incubated with various concentrations of bovine TSH (0, 0.001, 0.01, 0.1, 1, 10 and 100 mIU/ml) in the binding buffer (final volume, 0.1 ml) for 1 h at 37°C. Subsequent to the incubation, medium was removed and replaced with 0.1 M HCl. The cell extracts were dried in a vacuum concentrator and cAMP levels were determined using a cAMP Enzymeimmunoassay kit (Westang Biotechnology Co., Ltd. Shanghai, China) according to the manufacturer's protocol and expressed as pmol/ml. Each experiment was performed a minimum of three times.

Statistical analysis. Data are expressed as the mean \pm standard deviation, from three repetitions. Statistical analysis between the mutation and wild-type groups was performed using Student's t-test (SPSS 13.0; SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to represent a statistically significant difference.

Results

Identification of V87L mutation in TSHR gene. Sequence analysis of the DNA from the patients' thyroid tissue revealed a guanine-to-cytosine transversion at position 259 (Fig. 1). The aforementioned mutation results in an amino acid substitution of valine by leucine at codon 87 in the extracellular domain.

Functional studies of the novel mutant receptor. Immunoblot analysis revealed that following transfection of the ectopic

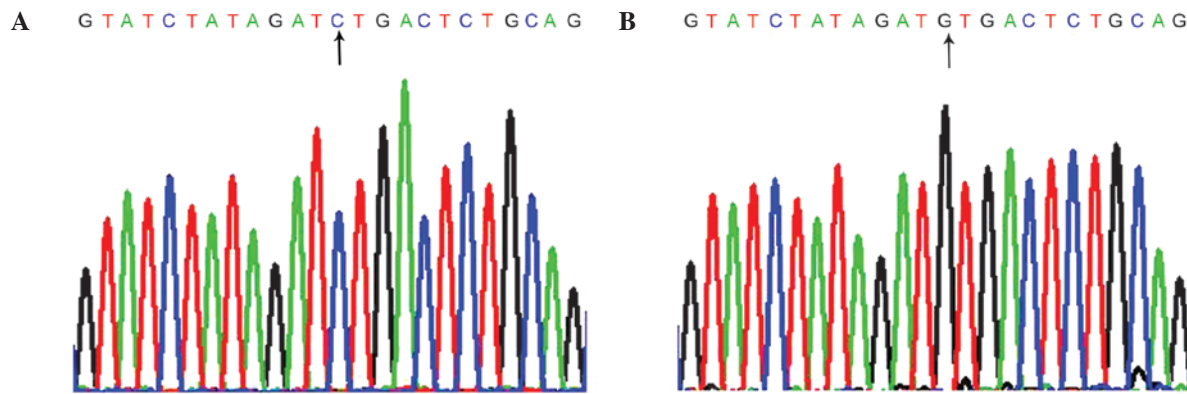


Figure 1. Sequence analysis of the DNA extracted from the patients' thyroid tissue. (A) The arrow indicates a single base exchange, resulting in the V87L mutation. (B) The corresponding sequence of the wild-type TSHR. TSHR, thyrotropin receptor.

expression plasmids, V87L and the wild-type TSHR were expressed in CHO cells. However, the V87L mutant displayed markedly lower expression levels compared with the wild-type TSHR (Fig. 2).

Competitive TSH binding studies indicate reduced binding activity of the mutant receptor. V87L-TSHR was shown to have a markedly (but not significantly) reduced TSH binding capacity (B/B_0 values) compared with that of the wild-type TSH receptor (Fig. 3).

Furthermore, the ability of the CHO cells transfected with mutant TSHR to produce cAMP in response to bTSH was significantly decreased. The maximum cAMP concentration did not exceed half of that in the cells transfected with the wild-type receptor, and the EC_{50} values for V87L-TSHR were lower compared with those for the wild-type receptor (Fig. 4). cAMP production in the V87L-TSHR-transfected cells showed a gradual increment with the increase of bTSH concentration, while the cAMP production in the wt group rose rapidly above a bTSH concentration of 0.01 mIU/ml, peaking at 10 mIU/ml, indicating the reduced function of the mutant receptor.

Discussion

The TSHR, located along the basolateral membranes of the thyroid follicular cells, belongs to the receptor superfamily of guanine nucleotide-binding protein-coupled receptors, possessing similar features to other members of the same family, including follicle-stimulating hormone and luteinizing hormone receptors, such as the typical structure of seven transmembrane domains. Upon ligand binding, receptor activation occurs, leading to increased intracellular cAMP and ultimately thyroid-cell proliferation and differentiation (15).

The TSHR gene has been defined as highly mutable (16). Mutations in the TSHR gene result in either gain or loss of the receptor function. Gain-of-function mutations are the cause of the following three syndromes: Familial nonautoimmune hyperthyroidism (FNAH), sporadic congenital nonautoimmune hyperthyroidism (SCNAH) and autonomous adenomas (AA) (17). FNAH is also termed hereditary toxic thyroid hyperplasia or autosomal dominant autoimmune hyperthyroidism. It is hereditary through a dominant activating mutation of the TSHR affecting all thyroid cells (18). SCNAH is a result

of germline neomutations affecting all thyroid cells (19). A large proportion of AAs are also caused by activating mutations of the TSHR; however, AA affects only one cell at the clonal origin of a benign tumor (20,21) and the majority of AA mutations are somatic (22). LOF mutations are predominantly recessively inherited and lead to the phenotype of TSH unresponsiveness (2), encompassing a wide spectrum of transient or permanent clinical and biochemical manifestations, ranging from complete resistance in nonautoimmune hypothyroidism with *in situ* normal-to-hypoplastic thyroid gland (23-28) or to partial resistance in nonautoimmune compensated hypothyroidism (2,7-10,29), defined as slightly elevated serum TSH associated with normal free thyroid hormone levels. Compensated hypothyroidism is also referred to as subclinical hypothyroidism (15). Somatic LOF mutations are rare (4).

The degree of TSH resistance depends on the severity of the impairment of the receptor function caused by the mutation and on the number of mutated alleles (30). In cases of both alleles carrying mutant receptors with complete lack of function, the typical result is severe congenital hypothyroidism with a hypoplastic thyroid gland (uncompensated TSH resistance). Less severe LOF mutations, predominantly recessively inherited, can manifest as mild/borderline forms of hypothyroidism, in which an appropriate increase in TSH serum levels can compensate for the reduced sensitivity of the thyroid (partially or fully compensated TSH resistance) (14).

The results of the present study were consistent with the hypothesis that an inactivating mutation of TSHR partially accounts for nonautoimmune subclinical hypothyroidism. The patient of the present study had thyroid hormone levels in the lower limit of normal range and increased serum TSH levels, while she tested negative for circulating TgAb and TPOAb. The sequence analysis of the DNA extracted from the patients' thyroid tissue revealed a guanine-to-cytosine transversion at position 259. This mutation resulted in an amino acid substitution of valine by leucine at codon 87 in the extracellular domain. The expression of mutant TSHR was a little lower than that in the wild-type group; this may be due to reduced transcription/translation, which requires confirmation in future studies. Reduced receptor expression may lead to decreased receptor density at the cell surface, which causes reduced competitive TSH binding activity. A previous competitive TSH binding study revealed reduced binding activity of the mutant receptor.

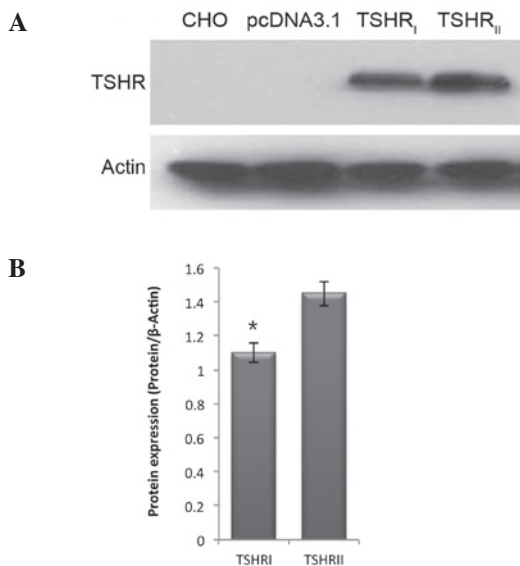


Figure 2. Western blot analysis of TSHR protein expression. (A) Bands correspond to TSHR and β -Actin. (B) Densitometry analysis of the bands from (A) normalized against β -Actin. Results were obtained from three independent experiments performed in triplicate. * $P < 0.05$ vs TSHR_{II}. CHO, CHO cells without transfection; pcDNA3.1, CHO cells transfected with pcDNA3.1 without TSHR fragment; TSHR_I, CHO cells transfected with V87L mutant; TSHR_{II}, CHO cells transfected with wild-type TSHR. TSHR, thyrotropin receptor; CHO, Chinese hamster ovary.

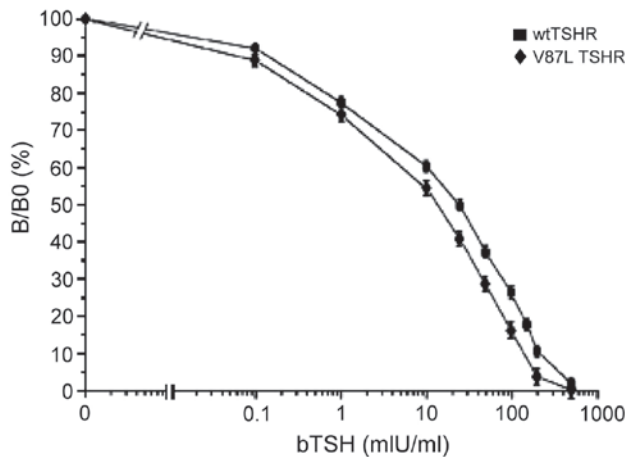


Figure 3. Competitive TSH binding activity of Chinese hamster ovary cells transfected with wtTSHR or V87L-TSHR. B/B0, binding in the presence of the competitor/binding in the absence of competitor (maximal binding); bTSH, bovine thyrotropin; wtTSHR, wild-type TSH receptor.

Decreased receptor expression may be a reason for the reduced TSHR activity, but following binding with TSHR, TSH functions through cAMP. The cAMP levels in cells transfected with mutant TSHR vector were decreased compared with those in cells transfected with wild-type TSHR, indicating that this mutation is an inactivating mutation. The maximum cAMP concentration in the mutant-transfected group did not exceed half of that in the wild-type receptor group, indicating a LOF for the mutated receptor by $>50\%$.

The results of the sequencing revealed the mutation to be a homozygous mutant. However, thyroid function was not completely lost in the patient, although more than half of

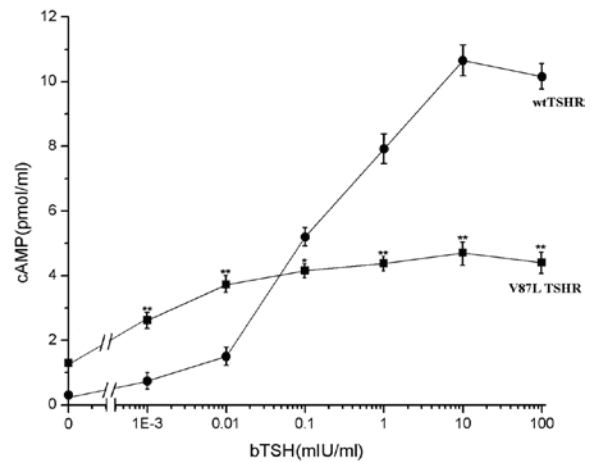


Figure 4. cAMP production of Chinese hamster ovary cells transfected with wtTSHR or V87L-TSHR. Results were obtained from three independent experiments performed in triplicate. * $P < 0.05$, ** $P < 0.05$ vs. wtTSHR cAMP, cyclic adenosine monophosphate; bTSH, bovine thyrotropin; wtTSHR, wild-type TSH receptor.

her thyroid function was lost. The patient's T4 levels may be maintained by an appropriate increase in serum TSH levels, indicating that the mutation of the TSHR did not severely impair the receptor function. Considering the TSHR gene in its entirety, this mutational site is a less harmful one. The patient's parents are deceased and she has no siblings. Her only child, a 34-year-old male, has a normal thyroid function (TSH, 2.5 mU/l), indicating that the mutation may be recessively inherited and not X-linked. It is therefore likely that the mutation is autosomal recessive inherited.

In conclusion, the present study revealed a novel TSHR mutation (V87L) in a Chinese woman with subclinical hypothyroidism, which, to the best of our knowledge, has not been previously reported. Decreased cAMP accumulation in cells transfected with V87L-mutated TSHR compared with those transfected with wild-type receptor suggested that the mutation is associated with LOF. The findings of the present study may provide valuable insight into the etiology of SH.

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