Autonomously Functioning Thyroid Nodule Associated with Thyrotoxic Periodic Paralysis

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Abstract. Thyrotoxic periodic paralysis (TPP) is mainly associated with Graves' disease but rarely with autonomously functioning thyroid nodule (AFTN). We herein report a case of AFTN associated with TPP in which the latter resolved after ^{131}I therapy for the former. We analyzed the genes encoding thyrotropin receptor (TSHR), the α -subunit of the stimulatory G protein ($\text{Gs}\alpha$), calcium channel CACNA1S and potassium channel KCNE3, and found that the patient does not carry the known mutations in these genes. Whereas the pathogenesis of TPP and AFTN remains to be understood, the present case suggests that ion channel defects responsible for familial hypokalemic periodic paralysis may not be associated with TPP, and that mutations in TSHR and $\text{Gs}\alpha$ genes may be less frequent in AFTN patients in the Japanese population.

Key words: Autonomously functioning thyroid nodule, Thyrotoxic periodic paralysis, TSH receptor, Ion channels

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THYROTOXIC periodic paralysis (TPP) is a potentially life-threatening complication of thyrotoxicosis characterized by acute and reversible episodes of muscle weakness and hypokalemia due to a massive intracellular shift of serum potassium [1]. This condition mainly affects male patients of Asian descent, particularly Chinese and Japanese [2–4]. While the pathogenesis of TPP remains unclear, its clinical features are similar to those of an autosomal dominantly inherited syndrome, familial hypokalemic periodic paralysis (FHPP). Defects in ionic channels have been identified as the cause of FHPP. Several mutations have been demonstrated in the human skeletal muscle genes encoding the voltage-gated calcium channel $\alpha 1$ subunit Cav1.1 (CACNA1S) [5–7], the MinK-related peptide 2 that coassembles with Kv3.4 (KCNE3) to form the

voltage-gated potassium channel [8], and the voltage-gated sodium channel Nav1.4 (SCN4A) [9, 10]. In contrast, such defects in these ion channels have only rarely been reported in patients with TPP [11–14]. Thus, the pathogenic role of ion channel defects in TPP remains under debate. Some studies suggested an association of certain human leukocyte antigen haplotypes with TPP [15, 16].

Thyroid stimulating hormone (TSH) is the main trophic factor that controls thyroid function and growth. TSH binds to its receptor (TSHR) and activates the α -subunit of the stimulatory G protein ($\text{Gs}\alpha$), leading to adenylate cyclase (AC) activation and subsequently cyclic AMP (cAMP) production. In autonomously functioning thyroid nodule (AFTN), which is characterized by autonomous, TSH-independent hypersecretion of thyroid hormones, gain-of-function mutations have been identified in TSHR and $\text{Gs}\alpha$ genes [17–20]. These mutations result in the constitutive activation of the AC-cAMP pathway, leading to cellular hyperfunction and proliferation. The frequency with which these activating mutations in the TSHR gene are found in AFTN is variable, but reaches up to

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80% [19]. In contrast, such mutations in the $G\alpha$ gene are reportedly less frequent [19, 20]. AFTN is a frequent cause of hyperthyroidism in Europe but very rare in Japan [21], and the frequency of the gain-of-function mutations in the TSHR gene and/or $G\alpha$ gene is reportedly very low in Japan [22, 23]. The reason for such differences remains unclear. The frequency of such mutations appears to be high among AFTN patients in iodine-deficient regions, whereas it is low among them in iodine-replete regions [19, 22, 23]. Thus, daily iodine intake might contribute to the different frequency of AFTN among countries.

While TPP is commonly associated with Graves' disease, it is also seen in other types of thyrotoxicosis such as destructive thyroiditis, AFTN and TSH-secreting pituitary adenoma [1, 24–27]. TPP associated with AFTN, however, is very rare as there are only a few case reports [24, 25]. Here we report a case with AFTN who suffered from attacks of TPP. In addition, we analyzed mutations in exons 9 and 10 of the TSHR gene and exons 8 and 9 of $G\alpha$ gene, which are frequently detected in AFTN from European countries [18–20], as well as mutations in CACNA1S and KCNE3, which are identified as the cause of FHPP [5, 6, 8].

Case Report

A 35-year-old Japanese man presented to a clinic because of severe muscle weakness in his legs in September, 1999. He was unable to walk, or even to stand without assistance. He had experienced frequent episodes of transient muscle weakness in his legs over the previous two years. He had no significant past medical history and took no medication. However, he was aware of heat intolerance and hyperidrosis at the age of 30. His family had neither the episodes of transient paralysis nor history of thyroid diseases. Physical examination revealed normal vital signs, normal sensation and cranial nerves, and symmetric flaccid paralysis of lower extremities. Neuromuscular disorders such as Guillain-Barré syndrome were suspected and thus he was referred to a neurology clinic for evaluation of the muscle weakness. His symptoms gradually resolved over a 12-hour period, during which serum potassium level was not measured. No abnormal findings were found in serum potassium, cerebrospinal fluid, electromyography and peripheral nerve conduction velocity, which were examined in the absence of paralysis. Because of elevated serum T4 and T3 levels (free T4 2.65 ng/dl; normal, 0.7–1.8 and free T3 6.07 pg/ml; normal, 2–4.5) and suppressed serum TSH (<0.05 μ IU/ml; normal, 0.3–3.5), he was referred to our clinic for the evaluation of thyroid function (Fig. 1). At his first

Fig. 1. Thyroid function tests and attacks of periodic paralysis. Normal ranges of serum total T4 (TT4, closed circle), free T4 (FT4, open circle) and TSH (closed triangle) are 4–12 μ g/dl, 0.7–1.8 ng/dl and 0.3–3.5 μ IU/ml, respectively.

visit, he was free from muscle weakness. On physical examination, he had fine finger tremor and a nodular goiter in the left lobe of the thyroid. Eye signs associated with Graves' ophthalmopathy were absent. Achilles tendon reflex was not accelerated. Blood pressure and heart rate were normal. Laboratory tests revealed suppressed serum TSH (<0.03 $\mu\text{IU/ml}$) accompanied by normal serum total T4 (9.6 $\mu\text{g/dl}$; normal, 4 – 12) and total T3 (1.48 ng/ml ; normal, 0.7 – 1.8). TSH receptor antibody (TRAb) was absent. Thyroid ^{123}I scintigraphy (Fig. 2A) revealed the accumulation of radioiodine in the left lobe but not in the right lobe. Thyroid ultrasonography (Fig. 2B) demonstrated an encapsulated nodule with increased vascularity in the upper portion and cystic changes in the middle to lower portion. The right lobe was atrophic. These findings led us to a diagnosis of AFTN. Additionally, TPP was highly suspected based on his history despite the absence of information on serum potassium levels during the paralytic attack. While he was in the state of subclinical hyperthyroidism, he was left untreated by his own choice. His thyroid function had been evaluated periodically for the following two years, during which he has been free from muscle weakness. However, he began to experience repeated transient periodic paralysis again in 2005. Recurrence of hyperthyroidism was confirmed by elevated serum free T4 and free T3 (2.1 ng/dl and 4.8 pg/ml , respectively) with decreased serum TSH (<0.03 $\mu\text{IU/ml}$). However, serum potassium levels during the paralysis attacks remained unknown since he always visited our clinic after the symptoms of weakness were resolved. He was unaware of myalgia and muscle weakness in the absence of paralytic attacks, suggesting that he did not suffer from thyrotoxic myopathy. Whereas his clinical course had been carefully followed without medication for several months, hyperthyroidism and periodic paralysis did not resolve. Accordingly, he received 296 MBq of ^{131}I in May, 2006. He experienced transient atrial fibrillation and thus received methimazole and aspirin for two months. Thereafter, his thyroid function has remained in the state of subclinical hyperthyroidism (free T4 1.2 – 1.7 ng/dl and TSH <0.03 $\mu\text{IU/ml}$) without antithyroid drugs, and he has been free from muscle weakness and arrhythmia.

Fig. 2. Thyroid ^{123}I scintigraphy (A) and thyroid ultrasonography (B).

Materials and Methods

Sequencing of the TSHR and Gsa genes

Thyroid cells were collected from the nodular lesion in the left lobe by fine needle aspiration biopsy; cytological examination revealed the absence of atypical cells, suggesting adenomatous goiter. Genomic DNA was isolated from the thyroid sample using a commercial kit (Promega, Madison, WI). The entire exons 9 and 10 of the TSHR gene and exons 8 and 9 of the Gsa gene were amplified using the primer couples shown in Table 1. The polymerase chain reaction (PCR) was performed in a 25 μl reaction volume containing 100 – 200 ng of genomic DNA, 10 pmol of each upstream and downstream primer, 1.25 units of Taq polymerase (Takara, Otsu, Japan), 0.2 mM each dNTP, and 2 mM MgCl_2 . All the reactions were started by an initial denaturation at 95°C for 5 min (except for exon 9 of TSHR at 93°C for 2 min 30 sec and exon 10 of TSHR at 94°C for 2 min), followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec (except for exon 9 of TSHR at 61°C for 30 sec) and extension at 72°C for 1 min [17, 18, 22]. Aliquots of PCR products were run in 1% agarose gel electrophoresis and visualized with ethidium bromide to verify their quality. PCR products were purified and then subjected to the sequencing reaction using a dye termi-

Table 1. Primer sequence for mutation detection in TSHR, Gs α , CACNA1S and KCNE3 genes.

Exon	Forward primer	Reverse primer
TSHR 9	5'tcatctcccaattaacctcagg3'	5'gcttccaatttcctctccac3'
TSHR 10 (residue 295–449)	5'ccttgagtccttgatgtgaatg3'	5'caggttgacatgagaaagc3'
TSHR 10 (residue 430–537)	5'ttcgtagtctgctggtc3'	5'caaccatgatggcacatg3'
TSHR 10 (residue 526–646)	5'ctggtagtccatcaccttc3'	5'-tgagaggctgttcagaatt3'
TSHR 10 (residue 635–stop)	5'tgttgatcttcaccgacttc3'	5'taagttcccctaccattgtg3'
Gs α 8	5'tactecagaccttgcctta3'	5'gtcaagaaccatgatctct3'
Gs α 9	5'agagatcatggtttcttgac3'	5'cagaagcaaagcgttctta3'
CACNA1S 11 (R528H)	5'ggagatcctgctggtggagtcg3'	5'tcctcaggaggcggatcgag3'
CACNA1S 30 (R1239H)	5'tccagcgcttcttcgcct3'	5'caggagggttcgcactcct3'
KCNE3 (R83H)	5'ttgctgtaactgtgggcag3'	5'atgcagtcacagcagagtt3'

TSHR; TSH receptor, Gs α ; the α -subunit of the stimulatory G protein, CACNA1S; calcium channel Cav1.1, and KCNE3; potassium channel Kv3.4.

nator cycle DNA sequencing kit (Applied Biosystems, Foster City, CA) and Applied Biosystems, Inc. Prism 310 DNA sequencer (Applied Biosystems). This study was approved by the institutional review board of Tohoku University School of Medicine and informed consent was obtained from the patient.

Screening of the mutation in ion channels

The presence of the mutation in CACNA1S or KCNE3 gene was screened by the endonuclease digestion of the PCR products as described previously [5, 6, 13]. Genomic DNA was extracted from white blood cells as mentioned above. DNA samples were amplified in volumes of 50 μ l, containing 1 μ g of genomic DNA, 10 pmol of each upstream and downstream primer, 2.5 units of Taq polymerase (Toyobo, Osaka, Japan), 0.2 mM each dNTP, and 2 mM MgCl₂. The primer sequences for mutation detection in the CACNA1S and KCNE3 genes are shown in Table 1. The amplifying cycles were an initial phase of 2 min at 94°C, followed by 35 cycles of a denaturation step of 30 sec at 94°C, an annealing step of 30 sec at 55°C and an extension step of 1 min at 72°C. Aliquots of PCR products were subjected to 1% agarose gel electrophoresis to verify the sizes and then purified with phenol chloroform. For the rapid screening of point mutations R528H and R1239H in the CACNA1S gene, we submitted the PCR products to overnight digestion at 37°C with *Bbv*I and *Nla*III endonucleases (New England BioLabs, Beverly, MA), respectively. The digestion products were run in 5% agarose gel electrophoresis and visualized with ethidium bromide. The mutation R528H disrupts a *Bbv*I restriction site and, as

a result, the affected allele shows a 77-base pair (bp) PCR product, whereas normal DNA yields two fragments of 33 and 44 bp [5, 6, 7]. The R1239H mutation creates an additional restriction site for *Nla*III in the PCR products (81 bp); thus the wide-type allele presents two fragments of 48 and 33 bp, whereas the mutant allele does three fragments of 48, 28 and 5 bp [6, 7]. Furthermore, to detect the CCC/CCT polymorphism at nucleotide 1564 and the GGC/GGT polymorphism at nucleotide 1551, we used *Bst*OI and *Hha*I endonucleases (New England BioLabs) and digested the PCR products used in the screening for the R528H mutation at 60°C for 3 hr and at 37°C overnight, respectively [11]. The digestion products were run in 5% agarose gel electrophoresis and visualized with ethidium bromide. The wild-type allele at nucleotide 1564 has two bands of 38 and 39 bp while the polymorphic allele abolishes the *Bst*OI site, producing a 77 bp band in homozygotes, or two bands of 38/39 and 77 bp in heterozygotes. *Hha*I digestion of the wild-type allele at nucleotide 1551 produces two bands of 51 and 26 bp while the polymorphic allele abolishes the *Hha*I site, producing a 77 bp band in homozygotes, or three bands of 26, 51 and 77 bp in heterozygotes.

For the detection of R83H mutation in the KCNE3 gene, the primers amplified a PCR product of 192 bp and this was digested with *Dra*III at 37°C overnight (New England BioLabs) [13]. The products were analyzed in 5% agarose gel electrophoresis with ethidium bromide staining. The G to A transition at nucleotide 340 creates a new *Dra*III restriction site in the products; the mutant product is cleaved to produce a fragment of 146 bp while the wild-type remains uncut [13].

Results

No mutations were identified in the exons 9 and 10 of the TSHR gene nor in the exons 8 and 9 of the *Gsα* gene. In addition, the patient did not harbor any of the studied mutations in the *CACNA1S* and *KCNE3* genes. The polymorphism GGC/GGT at nucleotide 1551 and the polymorphism CCC/CCT at nucleotide 1564 in the *CACNA1S* gene were not identified in the present case.

Discussion

Constitutively activating mutations of the TSHR have been identified in a number of patients with AFTN in European countries. The frequency of these mutations varies among reports but amounts to 50–80% [19–21]. In contrast, it has been reported to be very low in Japan [23]. However, Kosugi *et al.* analyzed the entire exon 10 of the TSHR gene and found the first activating mutation in the TSHR gene in a Japanese patient with AFTN [17]. Vanvooren *et al.* consistently identified four mutations in the TSHR gene in 10 Japanese AFTN patients and thus suggested that activating mutations in the TSHR gene may not be rare in the Japanese population [18]. In contrast to the TSHR gene, the incidence of the mutations in *Gsα* is reportedly low in Europe as well as in Japan [18–20, 22]. In the present report, we tested whether our patient harbored the mutations in exons 9 and 10 of the TSHR gene and those in exons 8 and 9 of the *Gsα* gene, in which the known hot spots for mutations exist [19, 28]. We did not detect any mutations in these genes. Whereas we did not examine other exons of TSHR and *Gsα* genes, our results suggest that oncogenic mutations of these genes might not play a major role in the development of AFTN in Japan. Further studies on a larger number of AFTN patients are clearly required to determine the actual prevalence of gain-of-function mutations in the TSHR and *Gsα* genes in the Japanese population since previous studies have analyzed these mutations in only a small number of AFTN cases [17, 18].

FHPP and TPP share common clinical features such as episodic attacks of muscle weakness, precipitating episodes such as ingestion of carbohydrate-rich meals, and decreased serum potassium levels during the attack [1]. It is well known that mutations in genes encoding skeletal muscle ionic channels such as *CACNA1S*,

SCN4A, and *KCNE3* are associated with FHPP [5–10]. In view of the similarities between FHPP and TPP, such mutations have been examined in TPP patients. Most studies have demonstrated that none of the TPP patients carry the known mutations in *CACNA1S*, *SCN4A*, and *KCNE3* genes [11, 13, 29, 30]. In line with these studies, we did not detect the known defects in *CACNA1S* and *KCNE3* genes, supporting the hypothesis that mutations in skeletal muscle ion channel genes associated with FHPP are not involved in the pathogenesis of TPP. In contrast, there are reportedly two TPP patients who carry such mutations; one patient of Portuguese descent with the R83H mutation in the *KCNE3* gene [12] and one pediatric Caucasian patient with the R672G mutation in the *SCN4A* gene [14]. However, the possibility that such mutations are only a coincidental finding in these TPP patients cannot be ruled out. Furthermore, it remains unclear whether the mutations in ion channel genes, if any, can explain the important clinical difference between FHPP and TPP, that is, TPP resolves completely following normalization of thyroid function. Whereas we did not find the polymorphism GGC/GGT at nucleotide 1551 nor the polymorphism CCC/CCT at nucleotide 1564 in the *CACNA1S* gene [11] in the present case, the significance of these polymorphisms remains to be determined. Since Na/K-ATPase activity is significantly higher in TPP patients than in uncomplicated thyrotoxicosis [31], its gene was analyzed in Chinese patients with TPP and no mutations were identified [31]. In addition, polymorphism in the β_2 -adrenergic receptor gene is not associated with TPP in Korean patients [32]. Thus, further extensive studies are clearly required to elucidate the mechanism of TPP. While the frequency of TPP remains largely unknown but is reportedly very low in Western countries, it is obviously high in Asian populations [1–3]. The reason for the difference remains undefined. Previous studies suggest an association of genetic factors such as certain human leukocyte antigen haplotypes with TPP [15, 16]. Interestingly TPP predominantly affects males despite the predominance of thyrotoxicosis in females. Because of androgen-induced stimulation of Na/K-ATPase activity [33], sex-related factors might play a role in the etiology of TPP.

TPP is a very rare complication of AFTN, and only a few cases have been reported to date [24, 25]. The reason for such rarity remains unknown. TPP is common in Asian populations; the incidence of TPP is reported-

ly 1.9% in Japan [2, 3]. In contrast, TPP is less common in western countries; its incidence is reportedly 0.1–0.2% in thyrotoxic patients [1]. By contrast, AFTN is one frequent cause (~30%) of hyperthyroidism in Europe, but uncommon (~0.3%) in Japan [21]. Such epidemiological differences may result in the very low incidence of TPP associated with AFTN. In addition to different ethnic origins of the patients, daily iodine intake could be related to the incidence of AFTN [34]. Alternatively, autoimmunity may play a role in the pathogenesis of TPP because of the predominant

association of TPP with Graves' disease [1].

In summary, we herein report a rare case of TPP associated with AFTN. The present case does not carry the known mutations in genes encoding for TSHR, *Gsα*, *CACNA1S* and *KCNE3*. Our results suggest that such mutations may not play a major role in the pathogenesis of AFTN and TPP at least in Japan. Further studies on a large number of patients with AFTN and those with TPP are clearly required to determine the incidence and the pathogenic role of such mutations.

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