CLINICAL STUDY

Oncogenic mutations in the thyrotropin receptor of autonomously functioning thyroid nodules in the Japanese population

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Abstract

Objective: Constitutively activating mutations of the thyrotropin receptor (TSHR) have been found in the majority of autonomously functioning thyroid nodules (AFTNs) in European patients. The reported frequency of these mutations varies among reports but amounts to 50–80%. To date, only one such mutation responsible for AFTNs has been identified in the Japanese population and the pathogenic role of such mutations in Japanese AFTNs has been questioned. In the present study, we evaluated the frequency of activating mutations in the TSHR and *G α s* in 10 Japanese AFTNs.

Design: Genomic DNA was extracted from fresh frozen tissue. The TSHR and the almost entire sequence of the gene coding for the α subunit of *Gs* have been amplified and sequenced.

Results: In sequence analysis, four mutations in the TSHR (T632A, I486M, M453T and L512R) were found. To complete our analysis, we searched mutations in the gene coding for the α subunit of *Gs*, in the samples negative for TSHR mutations. In one case a mutation (R201H) affecting GTPase activity was found.

Conclusions: If we focus on the solitary nodules, we obtain the same mutation proportion as in European patients (70%). The absence of TSHR and *G α s* mutations in a significant proportion of autonomous adenomas in multinodular goiters suggests that other causes may also play a role in the genesis of these lesions.

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Introduction

Under physiological circumstances, thyroid stimulating hormone (TSH) is the main factor that controls thyroid function and growth (1–3). TSH acts by binding to its receptor which is a member of the large family of G protein-coupled receptors. In human thyroid, the TSH receptor activates adenylyl cyclase via a *G α s* protein and at higher concentrations phospholipase C via *G α q*. The main second messenger of TSH action, in the human thyroid is cyclic AMP (cAMP) (2). Intracellular concentrations of cAMP control the maintenance of the differentiated phenotype of thyrocytes, the level of functional activity of the gland (trapping of iodide, secretion of thyroid hormones) and growth (3, 4). A chronic increase in this concentration is thus expected to cause toxic hyperplasia if all the thyrocytes of a gland are involved (4), or clonal expansion of the cell and an autonomous adenoma as a result of a

somatic mutation. Clinically, autonomous functioning thyroid nodules (AFTNs) are well documented. They are well defined encapsulated benign tumors which grow, metabolize iodide and secrete thyroid hormones independently of TSH. They are diagnosed at scintigraphy by a high localized uptake of radioiodide or Tc-99m pertechnetate, surrounded by barely or non-visible quiescent tissue. The low uptake of normal tissue results from low TSH serum levels, themselves a consequence of autonomous secretion of thyroid hormones by the nodule. AFTNs can be solitary or part of a multinodular goiter. In European patients, the main cause of this disease is mutations in the TSH receptor, increasing the constitutive activity of this receptor on adenylyl cyclase (5–8). The reported frequency of these mutations varies in different articles from 50–80% in Belgium and Germany (6, 9). These include studies in which only part of the TSH receptor (exon 10) has been sequenced. In Japan, a study including

45 cases reported only one TSH receptor mutation, but no increase in the basal cAMP level could be demonstrated in cells transfected with this mutated receptor (10). Since then, one TSH receptor activating mutation has been found in a Japanese patient (11).

Any other player of the cAMP cascade, if activated, would lead to the same result as an activated TSH receptor. To date, two mutations of $G_{\alpha s}$ have been identified; Q227L and R201H (in exons 8 and 9), which activate the protein, 'locking' it in its GTP-bound signaling conformation leading to adenylyl cyclase activation in a receptor independent way (gsp mutations). gsp Mutations have been found in 3–6% of AFTNs in Europe (6, 9). A Japanese study, Tanaka *et al.* (12), found only one case out of 38 studied. Although the discrepancies between the Japanese and the European studies may perhaps be explained by methodological differences (13), they raise the question whether the frequency of activating mutations in the TSH receptor and $G_{\alpha s}$ in AFTNs is actually much lower in Japan than in Europe.

Subjects and methods

Patients

Ten adenomas and their juxtanodular tissue were obtained from Japanese patients. Some characteristics of the patients and their adenomas are summarized in Table 1. The diagnosis was established by scintigraphy. As shown in Table 1, four proven solitary adenomas were studied, three with total suppression of contralateral uptake, hyperthyroidism and decreased TSH levels and one with partial suppression of contralateral uptake, euthyroidism and decreased TSH levels. Six multinodular goiters were studied: three with hyperthyroidism and total suppression of contralateral uptake and TSH levels, two with euthyroidism, partial suppression of contralateral uptake and TSH levels and one with euthyroidism, normal TSH levels and

partial suppression of contralateral uptake. The study was approved by the local ethical committee, and informed consent was obtained from all the participants before testing.

Sequencing of the TSHR and $G_{\alpha s}$ genes

Fresh tissue, obtained by surgery in Japan, was frozen and sent to Brussels. Genomic DNA was extracted from adenomatous tissue and from juxtanodular quiescent tissue. Briefly, the samples were incubated with 10 mg/ml proteinase K in 50 mM Tris-HCl pH 8.0, 100 mM EDTA, 100 mM NaCl and 1% SDS at 56 °C overnight, then phenol extracted and ethanol precipitated. Genomic DNA was finally resuspended in 500 µl TE pH 7.5 (10 mM Tris-HCl, 1 mM EDTA). All coding portions of the TSH receptor gene were sequenced using a total of 14 PCR amplified fragments. Except for exon 1, the entire $G_{\alpha s}$ gene was amplified using nine couples of primers. Table 2 summarizes the pairs of primers chosen to amplify the TSHR and $G_{\alpha s}$. Each PCR was performed in a final volume of 20 µl containing 200 ng DNA, 0.5 µl Taq DNA polymerase (BRL), 3 pmol of each primer, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin and 0.2 mM dNTPs. For the amplification of the $G_{\alpha s}$ exon 12, we used the Ampli Taq Gold (Roche). All the reactions were started by an initial denaturation at 93 °C for 2 min 30 s (except for: $G_{\alpha s}$ exon 8–9, 95 °C for 3 min; $G_{\alpha s}$ exon 10–11, 95 °C for 2 min 30 s; $G_{\alpha s}$ exon 12, 95 °C for 12 min) followed by 30 cycles (for the $G_{\alpha s}$ exon 6: 35 cycles). Annealing temperature was 54 °C for TSHR 1, 4, 5, 7; 48 °C for TSHR 2, 10 residues 635-stop; 52 °C for TSHR 3, 6, 8, 10 residues 295–344, 10 residues 430–537, $G_{\alpha s}$ exon 7; 61 °C for TSHR 9; 50 °C for TSHR 10 residues 322–449, 10 residues 526–646, $G_{\alpha s}$ exon 3, 4–5, 10–11, 12, 13; 58 °C for $G_{\alpha s}$ Exon 2,6 and 59 °C for $G_{\alpha s}$ exon 8–9.

PCR products were sequenced on both strands using the DNA Sequencing Kit (PE Applied Biosystems).

Table 1 Summary of characteristics of the 10 AFTNs investigated in this study.

Patient	Sex	Nodule (By echography)	Suppression of contralateral (By scintigraphy)	FT3 (pg/ml)	FT4 (ng/dl)	TSH level µU/ml	TSHR mutation	$G_{\alpha s}$ mutation
1	F	Multiple	+/-	3.2	0.9	1.2	—	—
2	F	Multiple	+	5	2.2	0.0	T632A	ND
3	F	Multiple	+	5.8	1.6	0.0	—	—
4	M	Multiple	+/-	3.4	1.1	0.2	I486M	ND
5	F	Multiple	+	3.3	1.7	0.1	—	—
6	F	Multiple	+/-	2.9	1.2	0.4	—	—
7	F	Solitary	+	5.8	2.2	0.0	—	R201H
8	F	Solitary	+	7.0	3.7	0.0	L512R	ND
9	M	Solitary	+/-	3.8	1.1	0.2	—	—
10	M	Solitary	+	4.9	1.5	0.1	M453T	ND
Normal values				2.2–4.3	0.8–1.5	0.7–4.3		

ND, not determined. Scintigraphy: +, total suppression of uptake in the contralateral lobe; +/-, partial suppression of uptake in the contralateral lobe. For TSH 0.0 means <0.1 µU/ml.

Table 2 Summary of the primers used for the PCR amplification of the TSHR and G α s.

Exon	Forward primer	Reverse primer
TSHR 1	5' M13D GAGGATGGAGAAATAGCCCCGAG 3' ¹	5' M13R CACTACTTCGGGCTGTTATTGAG 3' ¹
TSHR 2	5' M13D TAAGGTGAATTATTAGAAAAGC 3' ¹	5' M13R CTTGATAGAACACGTTTAGAGAA 3' ¹
TSHR 3	5' M13D GCAGAAATCCATGAGGGTTGT 3' ¹	5' M13R AGAAACCAGGCCCTCCCATTTG 3' ¹
TSHR 4	5' SP6 ACCCTGTGGCGTAAATGCATAT 3' ¹	5' M13R CCCGACCCAGGCTATACACCATT 3' ¹
TSHR 5	5' M13D AGTTTGACTACAGGTTGTCTTC 3' ¹	5' M13R GCTTTACTTATCTTCAACCTACC 3' ¹
TSHR 6	5' M13D TATTGTGTCCTGTTATTTAAGTGCATA 3' ¹	5' M13R GTACTCTATAGAGTATATATGATAAGG 3' ¹
TSHR 7	5' M13D TGGGATACATATGTGGGACCTG 3' ¹	5' M13R TGTGGGTCACACTAACTCTGG 3' ¹
TSHR 8	5' M13D TGGTCACATTTTATTCTGATATTTG 3'	5' M13R ATATTCCTTTTGTATGTCTTACTC 3'
TSHR 9	5' M13D TCATCTCCCAATTAACCTCAGG 3' ¹	5' M13R GCTTCCAATTTCTCTCCAC 3' ¹
TSHR 10 residues 295–344	5' M13D TGGCACTGACTCTTTTCTGT 3' ¹	5' M13R TGAGCGTTGTTATGAGTATCC 3'
TSHR 10 residues 322–449	5' M13D TGTGAATGCCTTGAATAGCC 3'	5' M13R CAGGTTGCACATGAGAAAGC 3'
TSHR 10 residues 430–537	5' M13D TTCGTTAGTCTGCTGGCTC 3'	5' M13R CAACCATGATGGCACATG 3'
TSHR 10 residues 526–646	5' M13D CTGGTATGCCATCACCTTC 3'	5' M13R TGAGAGGCTTGTTCAGAATT 3'
TSHR 10 residues 635stop	5' M13D TGTGATCTTCACCGACTTC 3'	5' M13R TAAGTTCCCCTACCATTGTG 3'
G α s 2	5' M13D GACCTCCCTGCCCAAAGT 3'	5' M13R CCCCAGGCCCCACCTATA 3'
G α s 3	5' M13D CTGATGGTTGAGGAATGTAG 3'	5' M13R CGGTCTGCCTCAGTTTC 3'
G α s 4-5	5' M13D CAGTACTCCTAACTGACATG 3'	5' M13R ATGGACACTGTGCTCAGGTG 3'
G α s 6	5' M13D TCAGGGTTTGAATGACAGTG 3'	5' M13R TTATGTGCTGATGGGTTGG 3'
G α s 7	5' TGCCATTGACTTAGTGCTGC 3'	5' CAGAAGTCAGGACACGGCAG 3'
G α s 8-9	5' M13D CTCCCCACAGAGGACTCTGAGCC 3'	5' M13R AGAGCGTGAGCAGCGACCTGATC 3'
G α s 10-11	5' M13D GGCTGTTTCGTAAGAAGC 3'	5' M13R GAACACCGCAATGAACAGC 3'
G α s 12	5' M13D CTAGCACCCAGCTCTGCTTG 3'	5' M13R CATGGAAGTGGGTAGCTGAAC 3'
G α s 13	5' M13D CATCAGGGATAGGGTGGTTC 3'	5' M13R CTTGTACAATTACGTTTCAC 3'

¹ Primers defined by de Roux *et al.* (14); M13D, M13R and SP6 correspond to universal sequencing primers (ABI).

Samples were loaded on an Applied Biosystems 373 Stretch Sequencing Instrument and analyzed using the Fatura and Sequence Navigator software (ABI).

Results

By sequencing the entire TSHR gene in 10 AFTNs from Japanese patients, four mutations were identified (Table 1). The only mutation L512A described in the Japanese population by Kosugi (11) was among the four mutations found. Three mutations detected (I486M, M453T and L512R) have already been reported in the European population and all enhance the constitutive activity of the receptor versus adenylate cyclase when expressed in eukaryotic cells (6, 9, 14, 15). The fourth T632A has been found in AFTN and cancer but its activating effect has not yet been demonstrated (9, 16), although the similar mutation T632I has been shown to confer constitutive activity to the receptor. All mutations were heterozygous and detectable only in the adenomatous tissue, not in the surrounding tissue. The samples which were negative for TSHR mutation were screened for G α s mutations. Although in general only exons 8 and 9 are investigated for mutations, we have completely sequenced the exons 2 to 13 of the G α s protein (gsp gene). The first exon could not be amplified by PCR because of the high GC content. One heterozygous G α s mutation in position 201, leading to an exchange of arginine to histidine was found in an adenoma. This activating gsp mutation has previously been identified in thyroid tumors (6, 9, 17).

Discussion

In the present study, we examined the incidence of TSHR and gsp mutations in a series of 10 autonomous thyroid adenomas from Japan. In a previous study, Takeshita *et al.* (10) examined 45 AFTNs in Japanese patients. They had found a TSHR gene alteration (deletion of three adenines which resulted in Asp 619 deletion) in only one case. This mutant did not show constitutive activation in transfection experiments. They concluded that there was no activating mutation of the TSHR in AFTNs in the Japanese population. However, the possibility that the AFTNs examined had TSHR mutations cannot be excluded because only a 167 base pair region was analyzed. Kosugi *et al.* who had identified a mutation responsible for AFTNs in the Japanese population sequenced the entire exon 10 of the receptor. However amino acid substitutions conferring constitutive activity of the TSHR have also been found elsewhere in the extracellular amino-terminal domain (18). We therefore sequenced the entire TSHR gene and identified four activating TSHR mutations among the 10 AFTNs from Japanese patients. We also searched for mutations in the gene coding for the α subunit of Gs. We analyzed not only the two hot spots classically described but almost the entire sequence from exon 2 to 13 of the protein G α s. One mutation at position 201, corresponding to one of the known hot spots affecting the GTPase activity, was discovered in one of the AFTN negative for TSHR activating mutations. The sequencing of the other coding portions of the G α s has not revealed any other mutation. Thus the incidence of

oncogenic mutations of proteins of the cAMP cascade in Japanese AFTN is not rare. Indeed, the frequency of such mutations in Europe is 50–80%. The discrepancy of the prevalence of TSHR mutations among AFTNs found in different populations was suggested to be associated with the different ethnic origins of the patients (10). It may in fact mainly be related to the completeness of the sequencing. It may also be related to the correspondence of the diagnosis in the different countries. It is also interesting that among the four solitary adenomas investigated, three demonstrated activating mutations of the TSH receptor or G α s, i.e. the same proportion as in Europe. These three adenomas are those in which all the criteria for the diagnosis were fulfilled: hyperthyroidism, low TSH levels and suppression of the uptake in the contralateral lobe. For the adenoma in which no mutation was found, the patient was not hyperthyroid, and both the contralateral uptake and the TSH levels were only partially suppressed. Only two activating mutations have been found in six hot nodules of multinodular goiters. One among the three patients with hyperthyroidism and suppression of contralateral uptake and TSH levels; one among the two who were not hyperthyroid and had only partial suppression of uptake and TSH levels; none in patient 1, who was euthyroid, had normal TSH levels and only partial suppression of contralateral uptake. The diagnosis of this latter adenoma is therefore doubtful. The Belgian and Italian statistics apply to solitary adenomas although mutations have been found in different nodules of the same toxic multinodular goiter, but less frequently (19–22). It should be noted that whereas single nodules are most often monoclonal, nodules of multinodular goiters are often polyclonal (23, 24). The absence of TSHR and G α s mutations in a significant proportion of autonomous adenomas suggests that other mutations may be found in the proteins of the cAMP cascade downstream of G α s or even in proteins of other cascades provided that the mutation leads to proliferation with conservation of differentiation (for example, constitutive activation of the IGF1 receptor, by IGF1, in transgenic mice leads to thyroid autonomy) (25).

The real difference between Japan and Europe is the low prevalence of autonomous adenomas in Japan, low prevalence has also been shown in the United States (26). This has been related to the high level of dietary iodide which has been shown in Denmark (27) and in Switzerland (28) to be inversely related to the incidence of thyroid autonomous adenomas.

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