CLINICAL STUDY

Prevalence of mutations in *TSHR*, *GNAS*, *PRKAR1A* and *RAS* genes in a large series of toxic thyroid adenomas from Galicia, an iodine-deficient area in NW Spain

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Abstract

Objective: Toxic thyroid adenoma (TA) is a common cause of hyperthyroidism. Mutations in the *TSH* receptor (*TSHR*) gene, and less frequently in the adenylate cyclase-stimulating G alpha protein (*GNAS*) gene, are well established causes of TA in Europe. However, genetic causes of TA remain unknown in a small percentage of cases. We report the first study to investigate mutations in *TSHR*, *GNAS*, protein kinase, cAMP-dependent, regulatory, type I alpha (*PRKAR1A*) and *RAS* genes, in a large series of TA from Galicia, an iodine-deficient region in NW Spain.

Design and methods: Eighty-five TA samples were obtained surgically from 77 hyperthyroid patients, operated on for treatment of non-autoimmune toxic nodular goitre. After DNA extraction, all coding exons of *TSHR*, *GNAS* and *PRKAR1A* genes, and exons 2 and 3 of *HRAS*, *KRAS* and *NRAS* were amplified by PCR and sequenced. Previously unreported mutants were cloned in expression vectors and their basal constitutive activities were determined by quantification of cAMP response element (CRE)-luciferase activity in CO7 cells transfected with wild-type and mutant plasmids.

Results: *TSHR* gene mutations were found in 52 (61.2%) samples, *GNAS* gene mutations in 4 (4.71%) samples and no *PRKAR1A* or *RAS* mutations were found. Only three previously unreported mutations were found, two affecting the *TSHR*, A623F and I635V, and one affecting the G-protein α -subunit (*Gs*), L203P. All mutant proteins showed higher CRE-luciferase activity than their wild-type counterparts.

Conclusions: TA in a hyperthyroid population living in Galicia, a Spanish iodine-deficient region, harbours elevated frequencies of *TSHR* and *GNAS* mutations activating the cAMP pathway. However, the genetic cause of TA was undetermined in 34% of the TA samples.

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Introduction

Non-autoimmune toxic nodular goitre is a common cause of hyperthyroidism in iodine-deficient regions (1, 2). Although several epidemiological risk factors for nodular goitres are well characterized (3), the molecular basis for most non-autoimmune toxic nodular goitres remains unknown (2). An exception is toxic thyroid follicular adenoma (TA) that is caused by activating mutations in the thyroid-stimulating hormone receptor (*TSHR*) gene (4) or, less frequently, in adenylate cyclase-stimulating G α -protein (*GNAS*) gene (5). In the absence of ligand binding, these mutations activate

the *TSHR* and the stimulatory *Gs* α , increasing intracellular cAMP levels and thus stimulating thyrocyte proliferation and thyroid hormone secretion (6). *TSHR* stimulation also activates the phospholipase C-diacylglycerol-inositol phosphate cascade (6), although the effects of this pathway in the pathogenesis of TA remain unknown (7).

Cyclic-AMP-dependent protein kinase (PKA) mediates the majority of effects of cAMP in many cell types and is required for the mitogenic stimulation of thyrocytes by thyroid-stimulating hormone (TSH) (8). Inactive PKA consists of a tetramer of two regulatory and two catalytic subunits. Binding of cAMP to the regulatory

subunits causes dissociation of the inactive enzyme into a dimer of regulatory protein bound to cAMP and two free catalytic subunits that phosphorylate specific substrates. *PRKAR1A*, the gene that codes for the PKA regulatory-subunit type-I α (RI α), is mutated in Carney complex type 1, a lentiginosis syndrome with multiple neoplasia that affects the thyroid gland, causing a spectrum of abnormalities from follicular hyperplasia to carcinoma (9). To date, however, mutations in *PRKAR1A* have not been implicated in toxic nodular goitres.

The RAS gene family (*HRAS*, *KRAS* and *NRAS*) encodes membrane-associated guanosine nucleotide-binding proteins that play a role in the transduction of signals arising from tyrosine kinase and G-protein-coupled receptors. Point mutations affecting the GTP-binding domain (codons 12 and 13) and the GTPase domain (codon 61) result in constitutive activation of the protein and tumour development (10). RAS mutations have occasionally been described in TA, suggesting a possible role in its pathogenesis (11).

The reported prevalence of mutations in the *TSHR* and *GNAS* genes in TA approaches 80% in some iodine-deficient regions in Europe (2). Toxic multinodular goitre is common in several regions of Spain where iodine deficiency is still found, but as yet the molecular basis of toxic nodular goitre in Spain does not appear to have been investigated. Our objective was to study the prevalence of mutations in *TSHR*, *GNAS*, *PRKAR1A* and *RAS* genes in a large series of TA from patients living in Galicia, an iodine-deficient region located in NW Spain (12).

Materials and methods

Thyroid tissue samples

Eighty-five thyroid samples, obtained from surgical specimens from 77 Galician native patients (19 males and 58 females) between January 2004 and December 2007, were studied. Surgery was indicated due to long-standing non-autoimmune toxic nodular goitre. Patient age (mean $\pm$ s.d.) was 53.6 ± 15.4 years (range: 22–78 years). Patients were from six hospitals of the Servicio Galego de Saude (SERGAS), the public healthcare network of Galicia. Three hospitals were University affiliated and three were first level hospitals without academic affiliation. All patients underwent a blood thyroid hormone test with serum TSH, free thyroxine (FT₄), free triiodothyronine (FT₃) and thyroid autoantibodies (TPOAb, TGAb) measured, a thyroid ultrasonography and a thyroid technetium 99 m (^{99m}Tc) scan. Patients were clinically diagnosed with clinical or subclinical hyperthyroidism, caused either by a non-autoimmune toxic solitary nodule or by a multinodular goitre, and referred for surgery. Pathological analysis revealed at least one TA in each surgical specimen. Care was taken to confirm that the location of each adenoma in the thyroid specimen

corresponded to a previously detected 'hot' area from the ^{99m}Tc scan. Tissue samples were taken from within the TA, from fresh (71 samples) or paraffin-embedded specimens (14 samples). The study was approved by the Ethics Review Panels of the Consellería de Sanidade (Xunta de Galicia, Spain) and the University of Santiago de Compostela.

Genetic studies

DNA samples were extracted using REALPURE Extraction DNA kit (Durviz, Valencia, Spain). Paraffin-embedded thyroid tissue was deparaffinated in xylene and a graded series of ethanol prior to DNA extraction. Out of 21 samples, 7 embedded in paraffin were rejected because of non-adequate DNA for PCR amplification.

TSHR gene coding exons and exons 2 and 3 of *HRAS*, *KRAS* and *NRAS* genes were directly sequenced in the 85 TA tissue samples. In samples that were found to have no mutations in *TSHR* and *RAS* (28 nodules), *GNAS* coding exons were sequenced completely and, in samples that had no mutations in *TSHR*, *RAS* or *GNAS* genes (24 nodules), *PKAR1A* exons 2–11 were sequenced. Exons and adjacent intron fragments of 50–100 bp were amplified by PCR (Tables 1 and 2 show oligonucleotide sequences; PCR conditions are available on request) and sequenced in an ABI PRISM 3100 (Applied Biosystems, Foster City, CA, USA) capillary electrophoresis apparatus. Where necessary, mutations were confirmed by enzyme restriction analysis or by fragment cloning into a pGEM-T TA cloning vector (Promega) followed by direct sequencing.

Clonality (AR) assay

Clonal origins of the TA tissues were analyzed by an X-chromosome inactivation assay performed on the human androgen receptor gene, *AR*, as described by Allen *et al.* with minimal modifications (13). DNA (100 ng) per sample were digested for 16 h at 37 °C in 20 μ l reactions with the methylation-sensitive HhaI (10 U) and HpaII (100 U) restriction enzymes (New England Biolabs, Hertfordshire, UK). Digestion efficacy was checked in parallel with a positive control DNA. A PCR (50 μ l) to amplify the polymorphic region inside *AR* exon 1 was performed with 20 ng digested DNA (PCR conditions and oligonucleotide sequences available on request). PCR products were loaded into a capillary electrophoresis apparatus (ABI PRISM 3100, Applied Biosystems) to separate each allele by fragment size. GeneScan – 500 LIZ Size Standard (Applied Biosystems) was included as an internal standard for the size fragment analysis. Allele intensity was calculated as described by Kopp *et al.* (14). A corrected ratio ((digested allele 1/allele 2)/(non-digested allele 1/allele 2)) value equal to or higher than three was chosen as the cut-off value for clonality, indicating that one of the

Table 1 Oligonucleotide sequences for PCR amplification of *TSHR* and *GNAS* coding exons.

Exon	Forward oligonucleotide	Reverse oligonucleotide
<i>TSHR</i>		
10	5'-GTCATGAGCCACTGCGCC-3'	5'-GGTTGAAGTTCATCGGACTTGG-3'
	5'-CCCAGGAAGAGACTCTACAAGCT-3'	5'-GAAACCAGCCGTGTTGCAC-3'
	5'-CCATCGACTGGCAGACAGG-3'	5'-CGGATTTTCGGACTGTGATGT-3'
	5'-ACATAGTTGCCCTTCGTCATCGT-3'	5'-GCTGTTCTTTGGAGGAACCC-3'
	5'-GCCTTCCAGAGGGATGTGTTTC-3'	5'-CCATGAAACATTGAAACATCGC-3'
9	5'-CATCTCCCAATTAACCTCAGGC-3'	5'-CAAACCAGGAAGCATCTTCCC-3'
8	5'-AGCCCTTGGAGCCCTC-3'	5'-CTGCTTTTGTGCACTTCG GG-3'
7	5'-CCAACATATTGTGAAAACGTCA-3'	5'-AATATAACTGCCATTGATTTATGC-3'
6	5'-TCCATGAGGGTTGTACATGT-3'	5'-CAATCAAGAAACAGCTCCC-3'
5	5'-GGTACCCTGTGGCGTAAATG-3'	5'-CCTGGACCACATCATAGG-3'
4	5'-TTAGGGAAGGTGTTGGGAG-3'	5'-TTATCTTCAACCTACCCTCATGA-3'
3	5'-GCGGTTAAGAAGGGTCAGTG-3'	5'-TGGATGGTCTGTAAACATATGAAA A-3'
2	5'-TGGGACCTGAAAACCTT TATG-3'	5'-TCTGGCGTGGAGAGTCT-3'
1	5'-GGGACTTGACAGAACGCTT TAC-3'	5'-GTGCTCAAGGCAAGAAGATA-3'
<i>GNAS</i>		
1	5'-ATGGGCTGCCTCGGGAACAG-3'	5'-TTACCCAGCAGCAGCAGGCCA-3'
2	5'-AAATGCCTCCTCATAACTGA-3'	5'-GCCCCACCTATACTTCTAAAG-3'
3	5'-ATGGTTGAGGAATGTAGAGAGACTG-3'	5'-CAGTATGATCTTCATGTTTGTG-3'
4-5	5'-ATGAAAGCAGTACTCCTAACTGA-3'	5'-TGGATGCTCCTGCCATGTG-3'
6	5'-ATTAGTTCAAGCTCTTGCCCTTC-3'	5'-TTGTCTGTTTATGTGCTGATGG-3'
7	5'-TGCTGCATAACTGTGGGACG-3'	5'-GTAGTTTGAAAGAGGGCTCAG-3'
8-9	5'-CCTCCCCACAGAGGACTCTGAGCC-3'	5'-AGAGCGTGCGCAGCGACCCTGCTC-3'
10	5'-TGTTAGGGATCAGGGTCGCTG-3'	5'-AACAGTGCAGACCAGGGCCTCCTG-3'
11	5'-CAGGAGGCCCTGGTCTGCACTGT-3'	5'-AGAACCACCGACCTGAACAGCC-3'
12	5'-AGACTTCAGGAGCTACAGAGA-3'	5'-AGAGGAGGAACAAGAGAGGAA-3'
13	5'-CATCAGAGGCTGGCTGACAGCC-3'	5'-AAGGCTTTAATTAATTGGG-3'

parental alleles represented 75% or more of the alleles present in the sample (14).

Construction of *TSHR* and *GNAS* plasmid vectors

The two new *TSHR* mutants were constructed using a *pSVL-TSHR-WT* (a gift from S Refetoff, Thyroid Study Unit, University of Chicago, IL, USA) as a backbone. Exon 10 DNA fragments of 1030 bp were PCR amplified from thyroid tissue samples carrying those mutations. After gel purification (Qiaex II Gel Extraction Kit, Qiagen GmbH), fragments were subcloned into a *pGEM-T* TA cloning vector (Promega), transformed into *Escherichia coli* JM109 and verified by direct sequencing. Fragments of 567 bp carrying the mutations were excised from a *pGEM-T* TA cloning vector with *Bsu* 36I and *Bst* EI restriction enzymes (New England Biolabs) and subcloned into the corresponding sites of the *pSVL-TSHR-WT* vector.

For the new *GNAS* mutant, a plasmid vector was obtained by site-directed mutagenesis on a *pSVK3-GNAS-WT* vector constructed as follows. Total RNA was obtained from a surgically excised normal thyroid tissue specimen and retrotranscribed using M-MLV reverse transcriptase (Invitrogen), 5 µM random hexamers and 2 mM deoxynucleotides (Ecogen, Barcelona, Spain) in a 20 µl reaction volume. A *GNAS* cDNA fragment of 1158 bp was PCR amplified using 5'-GTCGACATGGGCTGCCTCGGGAACAG-3' as a forward primer and 5'-CAGCTGTTGGGGGTTCCTTCTTCGA-3' as a reverse primer, both with a *Sall* restriction enzyme site

(underlined sequence) located at the 5' end. The fragment sequence showed that amplified cDNA corresponded to a *GNAS* transcript encoding a short form of $G\alpha$, which lacks 15 amino acids as a result of alternative splicing of exon 3, and has an extra serine residue due to an alternative splice acceptor site for exon 4 of the *GNAS* gene (15). After gel purification, the same fragment was inserted into a *pGEM-T* TA cloning vector, transformed into *E. coli* JM109 and verified by direct sequencing. The *GNAS* insert was excised from the *pGEM-T* TA vector by *Sall* restriction enzyme (New England Biolabs) and cloned into a *pSVK3* vector (a gift from Michael A Levine, Children's Hospital Cleveland Clinic, OH, USA) previously linearized with *Sall*. Finally, the construct was sequenced to confirm the correct insertion of the *GNAS* coding transcript into the *pSVK3* vector. Site-directed mutagenesis (QuickChange II site-directed mutagenesis kit, Stratagene, Madrid, Spain) was subsequently used to generate the new *pSVK3-GNAS* mutant. Primer sequences and PCR conditions are available on request.

Cell culture and transfection studies

Constitutive activities of the new *TSHR* and adenylate cyclase-stimulating $G\alpha$ protein mutants were investigated in co-transfection studies with a cAMP response element (CRE)-luciferase reporter plasmid (a gift from Dr Peter Syapin, Pharmacology Department, TexasTech University). COS-7 cells (simian virus 40-transformed African green monkey kidney fibroblast) were grown at

Table 2 Oligonucleotide sequences for PCR amplification of *PRKAR1A* exons 2–11 and exons 2 and 3 of *RAS* genes.

Exon	Forward oligonucleotide	Reverse oligonucleotide
<i>PRKAR1A</i>		
2	5'-TACAAGCATGTGTGTGTTTTTTC-3'	5'-TCACAATCACCTCATCATCTCC-3'
3	5'-ATTGGAACATGAGAGTGCCAGC-3'	5'-GGATGAAGTTCCACCCTGTTTT-3'
4–5	5'-GCAGGTTGCAAACGTGAAA-3'	5'-CGCCTCCTCTCCCGTAAC-3'
6	5'-GCTTCTAACTTTTTTACCCTCTTT-3'	5'-AATCACTATTCTTATTGCTCGGAA-3'
7	5'-GGTTTGAGGGTTTTTAACATTTAAG-3'	5'-TCACACTCTCAAACACCATGGT-3'
8	5'-ACAAGCTTTGACTTTCTGGTTTT-3'	5'-TCCCAAGTCCATCCAATTCTA-3'
9	5'-CAGCACCAAATAATACAGAGCAG-3'	5'-CCCTCTTAGAGCGTACAACTTTT-3'
10	5'-TTGCATAGGATGTTTAAGGTGC-3'	5'-TCAGGAGAAGACAAAATTATGGAA-3'
11	5'-TCATAGAAGTTAGCCTGTTACCCAT-3'	5'-TGGCGCTGCAAGTAGGG-3'
<i>HRAS</i>		
2	5'-CTGAGGAGCGATGACGGAAT-3'	5'-CTGGCACCTGGACGGC-3'
3	5'-GGGTCCCTGAGCCCTGTC-3'	5'-CTGGCCCCACCTGTGC-3'
<i>KRAS</i>		
2	5'-ATTATAAGGCCTGCTGAAATGA-3'	5'-GAAACCCAAGGTACATTTTCAGATA-3'
3	5'-CTGTGTTTCTCCCTTCTCAGG-3'	5'-GGCATTAGCAAAGACTCAAAAA-3'
<i>NRAS</i>		
2	5'-GCTCGCCAATTAACCCTGATT-3'	5'-GACAGGATCAGGTCAGCGG-3'
3	5'-AGCATTGCATTCCCTGTGGT-3'	5'-TGTGGTAACCTCATTTCCCCA-3'

100% confluence in 100×20 mm polystyrene tissue culture dishes (Falcon, Franklin Lakes, NJ, USA) in DMEM (Sigma–Aldrich) containing 10% foetal bovine serum (Sigma–Aldrich) and 50 µg/l gentamicin (Sigma–Aldrich), at 37 °C in 100% humidity and 10% CO₂. For transient transfections, cells were transferred to 24-well culture plates (~50 000 cells/well; Sarstedt, Barcelona, Spain) and grown in 500 µl of the same medium until they reached 70–80% confluence. On the day of transfection, the medium was removed, cells were washed twice with PBS and to each well 500 µl fresh medium was added, followed minutes later by Eugene 6.0 Reagent (Roche; 1.5 µl/well), CRE-luciferase reporter plasmid (250 ng/well), together with *pSVL-TSHR* wild-type (250 ng/well) or mutant expression (250 ng/well) vector, or *pSVL-TSHR* wild-type (50 ng/well) plus 50 ng/well *pSVK3-GNAS* wild-type or mutant. After 48 h cells were harvested and luciferase activity was determined using a Luciferase Assay System (Promega) and luminometer (Junior LB 9509, Berthold, Germany). Each plasmid was transfected in quadruplicate and each experiment repeated at least twice.

Statistical analysis

Luciferase values (mean ± s.d.) were compared by Student’s *t*-test using SPSS, Chicago, IL, USA 12.0 for Windows (SPSS). *P* < 0.05 was considered statistically significant.

Results

All patients had low or suppressed TSH (normal range: 0.41–4.94 mUI/l). Serum FT₃ was elevated in 72% (normal range: 3.89–6.60 pmol/l) and FT₄ was elevated in 32% (normal range: 10.94–21.75 pmol/l) of patients. A solitary toxic adenoma was found in 47 (61%) patients

and a multinodular goitre with toxic adenoma(s) was found in 30 (39%) patients. Average nodule diameter was 3.90 cm (range: 1.7–7.5 cm). A papillary thyroid carcinoma was incidentally found in thyroid surgical specimens from eight (10%) patients, with a diameter ranging from 0.1 to 1.5 cm. None of the papillary thyroid carcinomas were found in hot nodules.

Mutations were found in 56 (65.88%) samples (Table 3). Fifty-two samples (61.2%) had mutations located in the *TSHR* gene and four samples (4.71%) had mutations in the *GNAS* gene, but no samples were found to have mutations in either *RAS* or *PRKAR1A* genes. Nine patients each presented two toxic adenomas and, in two of those patients, there was a different *TSHR* mutation in each of the two nodules.

Although the number of women was higher than men, no differences were found in the mutation rate by gender (68.9% women versus 62.5% men).

TSHR gene mutations were located in exon 9 (2/56, 3.57% total) and exon 10 (50/56, 89.29% total). Twenty-six amino acids of the *TSHR* were affected by these mutations (Table 3). The majority of mutations were located in the sixth transmembrane segment and the third intracellular loop; serine²⁸¹ was the only amino acid affected in the extracellular domain of the *TSHR*; each of the three extracellular loops of the *TSHR* were affected by one mutation (I486F, I568T and V656F); no mutations were found in the fourth transmembrane segment. As shown in Table 3, mutations were mostly transversions or transitions; only one mutation was caused by a triplet deletion (Δ619) and the other was caused by substitution of two bases within a triplet (GCC→TTC A623F). The most frequent mutation was M453T (ten samples) located in the second transmembrane segment, followed by I568T (four samples) in the second extracellular loop of the *TSHR*. Mutations A623F and I635V had not been previously reported as naturally occurring mutations in the *TSHR* gene.

Table 3 Thyroid-stimulating hormone receptor (TSHR) activating mutations in 85 toxic thyroid adenomas from Galicia, an iodine-deficient region with endemic goitre located in NW Spain.

Amino acid	Base substitution	Mutation position	Pathology	N
S281N	AGC → AAC	ECD	MNG-TA	1
S281T	AGC → ACC	ECD	MNG-TA	1
S425I	AGT → ATT	1st TMH	TA	2
G431S	GGC → AGC	1st TMH	TA	1
M453T	ATG → ACG	2nd TMH	MNG-TA (3)/TA (7)	10
M463V	ATG → GTG	2nd TMH	MNG-TA	1
I486F	ATC → TTC	1st ECL	MNG-TA (1)/TA (2)	3
L512R	CTG → CGG	3rd TMH	MNG-TA (1)/TA (2)	3
L512Q	CTG → CAG	3rd TMH	TA	1
I568T	ATC → ACC	2nd ECL	MNG-TA (2)/TA (2)	4
V597F	GTC → TTC	5th TMH	TA	1
V597L	GTC → CTC	5th TMH	TA	1
D619G	GAT → GGT	3rd ICL	MNG-TA (2)/TA (1)	3
Δ619	GAT	3rd ICL	TA	1
A623V	GCC → GTC	3rd ICL	TA	1
A623I	GCC → ATC	3rd ICL	TA	1
*A623F	GCC → TTC	3rd ICL	TA	1
L629F	TTG → TTC	6th TMH	MNG-TA	2
I630L	ATC → CTC	6th TMH	TA	1
F631L	TTC → CTC	6th TMH	MNG-TA (1)/TA (2)	3
T632I	ACC → ATC	6th TMH	MNG-TA (1)/TA (2)	3
D633E	GAC → GAA	6th TMH	MNG-TA (1)/TA (1)	2
D633Y	GAC → TAC	6th TMH	MNG-TA	1
D633H	GAC → CAC	6th TMH	TA	2
*I635V	ATA → GTA	6th TMH	TA	1
V656F	GTT → TTT	3rd ECL	TA	1

N, number of mutations; ECD, extracellular domain; TMH, transmembrane domain; ECL, extracellular loop; ICL, intracellular loop; MNG-TA, toxic adenoma in a multinodular goitre; TA, toxic adenoma alone. Numbers in parenthesis indicate number of mutations. New mutations are marked by *.

Gsα protein mutations detected were: R201H and a previously unreported L203P, both in exon 8 of *GNAS*, and Q227E and Q227H located in *GNAS* exon 9.

Three non-synonymous polymorphisms were found in the *TSHR* gene: amino acid 36 (aspartic acid → histidine, D36H, G/CAC (0.977 G/G homozygous, 0.023 G/C heterozygous)), amino acid 52 (proline → threonine, P52T, C/ACC (0.918 C/C homozygous, 0.082 C/A heterozygous)) and amino acid 727 (aspartic acid → glutamic acid, D727E, GAC/G (0.919 C/C, 0.081 C/G)). D36H was associated once with L512R and once with D619G *TSHR* gene mutations. P52T was associated once with T632I, once with D633E and once with D633H. D727E was associated once with G431S, M453T, V597F and F631L. Allele localization of polymorphisms and associated mutations was not determined.

Four synonymous polymorphisms were found in the *GNAS* gene, located at: isoleucine 131, ATT/C (0.400 T/C heterozygous, 0.300 T/T homozygous, 0.300 C/C homozygous), proline 144, CCC/T (0.940 C/C homozygous, 0.060 C/T heterozygous), glycine 185, ATC/T (0.940 C/C homozygous, 0.060 C/T heterozygous), asparagine 371 (0.940 C/C homozygous, 0.060 C/T heterozygous). A non-synonymous polymorphism was also observed at amino acid 322 (glutamic acid →

aspartic acid, E322D, GAG/T, (0.940 G/G homozygous, 0.060 G/T heterozygous)).

One synonymous polymorphism was found in the *PRKARIA* gene, at alanine 29, GCG/A (0.830 G/G homozygous, 0.170 G/A heterozygous).

The clonality assay was performed on 23 informative samples. Of these samples, 6 (26%) were found to be monoclonal and 17 samples (74%) polyclonal. Two monoclonal (2/6, 33.3%) and ten polyclonal samples (10/17, 58.8%) had mutations in either the *TSHR* gene or *GNAS* gene.

The three new identified mutations, *TSHR* A623F and I635V, and Gsα protein L203P showed statistically significant higher basal constitutive cAMP activity than their counterpart wild types (Fig. 1).

Discussion

Galicia is an iodine-deficient region located in NW Spain with a high prevalence of toxic multinodular goitre. Activating mutations in the *TSHR* (4) and *GNAS* (5) genes are frequently found in toxic non-autoimmune multinodular goitre, accounting for nearly 80% of cases

Figure 1 Basal constitutive cAMP activity of TSH receptor (A) and Gsα protein (B) previously unreported mutants vs wild-types. COS7 cells were co-transfected with 250 ng/well of either wild-type or mutant plasmid and, either 250 ng/well of a cAMP response element (CRE)-luciferase plasmid for TSH receptor experiments, or 50 ng/well of TSH receptor wild-type and 50 ng/well of *GNAS* wild-type or mutant. After 48 hr CRE-luciferase was measured. Values from wild-types are shown as 100%. *TSHR* and *GNAS* mutants showed statistically-significant higher basal luciferase values than wild-types, indicating a higher constitutive cAMP activity for the mutants.

in some European series (16–24). In agreement with previous studies, a high prevalence of mutation in the *TSHR* gene was found in our TA series from Galician patients. All mutated *TSHR* showed higher basal cAMP levels than the wild-type *TSHR* (see <http://gris.ulb.ac.be>, <http://innere.uniklinikum-leipzig.de/tsh/>).

Additionally, three non-synonymous polymorphisms were found in the *TSHR* gene. D36H and P52T, in the extracellular portion of the *TSHR*, have been previously reported in European patients with Graves' disease and in healthy controls (25). Neither D36H nor P52T have been shown to have any differences either in *TSHR* binding (26, 27) or basal cAMP production level (28), compared with the *TSHR* wild-type. The third polymorphism that we found, D727E, showed a frequency close to the one reported in German patients (28). The D727E polymorphism is located within the carboxy terminal tail of the *TSHR* and in one study has been associated with non-autoimmune toxic multinodular goitre (29). A subsequent study, however, provided evidence against such an association (28). Functional studies have shown no differences in basal and TSH-stimulated CRE-luciferase activities between D727E and wild-type *TSHR* (20, 30, 31). Interestingly, *in vitro* studies performed with a double mutant A593N/D727E showed higher constitutive activity than D727E, although the double mutant had 2.3-fold lower constitutive activity than the A593N mutant alone (31). This finding suggests that constitutive activities of mutant *TSHR* could be modified by the presence in the same *TSHR* gene allele of D727E or other non-synonymous polymorphisms.

Two previously unreported mutations were located in the *TSHR*, A623F and I635V. Alanine⁶²³ is located in the third cytoplasmic loop, which plays a key role in the interaction of the *TSHR* with G-proteins. Mutations by isoleucine (4), valine (17) and serine (32) have previously been reported as naturally occurring activating mutations in TA. Substituting alanine⁶²³ for glutamic acid or lysine results in loss of TSH-stimulated inositol phosphate formation but does not increase basal cAMP accumulation (33). In the present study, the new mutation A623F showed an increased basal cAMP-mediated CRE-luciferase activity compared with the wild-type *TSHR*, indicating that substitution of alanine⁶²³ for phenylalanine is an activating mutation. Phenylalanine is a highly hydrophobic amino acid, suggesting that activating mutations in alanine⁶²³ are conservative (17). Mutations at isoleucine⁶³⁵ had not previously been reported for the *TSHR*. Isoleucine⁶³⁵ is located in the sixth transmembrane domain, and it is probable that a substitution of isoleucine for valine, both that are also highly hydrophobic amino acids, modifies the relative positions of the helices thus causing an increase in constitutive activity of the receptor.

The fact that in 85 samples of TA only two new *TSHR* gene mutations were found suggests that most naturally occurring activating mutations in the *TSHR*

have now been described (see <http://gris.ulb.ac.be>, <http://innere.uniklinikum-leipzig.de/tsh/>). There are, however, also several artificially created activating mutations located in amino acid residues where naturally occurring mutations have not been found to date (<http://gris.ulb.ac.be>). This raises the question of why naturally occurring activating mutations do not occur in some *TSHR* amino acids that have the capacity to constitutively activate the receptor.

The most frequent mutations in our series were C→T and T→C transitions and G→T transversions. CpG dinucleotides undergo TG transversions at high frequency (34). Since methylated CpG islands are prone to mutation due to deamination of 5-methylcytosine (35), we performed an *in silico* analysis (Methyl Primer Express Software 1.0, Applied Biosystem) of the *TSHR* gene coding sequence that showed a hypothetical 760 bp CpG island inside exon 10. Of the mutations we studied, only the T632I mutation was located in this hypothetical methylated CpG island, suggesting that deamination of 5-methylcytosine is not a common mutagenic mechanism in TA (36). Replication slippage in quasi-palindromic DNA sequences causes frameshift and base-substitution mutations (37). A computational analysis of the *TSHR* gene using Mfold version 3.2 (<http://mfold.bioinfo.rpi.edu>) indicates that imperfect DNA complementarity is not a cause for the high frequency of M453T mutation. Oxidative damage caused by an increase in H₂O₂ generation in thyroid glands under conditions of iodine and selenium deficiency could be a major cause for the high mutational rate at identified codons (38).

Activating *GNAS* mutations were found at much lower frequency than *TSHR* mutations, as shown by others (19, 23, 39, 40). Previous to our study, however, activating *GNAS* mutations causing TA had only been found at *Gsα* residues arginine²⁰¹ or glutamine²²⁷, which also cause growth hormone-secreting adenoma, corticotroph adenoma, non-functional pituitary adenoma, adrenal adenoma or macronodular adrenal hyperplasia, McCune–Albright syndrome and fibrous dysplasia of the bone (15). *Gsα* arginine²⁰¹ is the site at which cholera toxin catalyses ADP-ribosylation inhibiting *Gsα* subunit intrinsic GTPase activity. As a result of reduced GTPase activity, *Gsα* remains in its active GTP-bound form causing an unregulated activation of adenylyl cyclase (41). Replacement of arginine²⁰¹ by histidine decreases intrinsic GTPase activity and leads to activation of adenylyl cyclase (41). *Gsα* residue glutamine²²⁷ belongs to the protein guanine nucleotide binding domain (42). Replacement of glutamine²²⁷ by glutamic acid or histidine decreases GTP hydrolysis by slowing the switch mechanism that mediates the conformational transition between the GTP- and GDP-bound forms, leading to activation of adenylyl cyclase (42). No activating *GNAS* mutations have previously been reported in *Gsα* residue leucine²⁰³, located near arginine²⁰¹. A naturally occurring substitution of

leucine²⁰³ by proline was found in one TA from our series and *in vitro* analysis showed an increase in constitutive CRE-luciferase activity in cells co-transfected with *pSVL-TSHR* wild-type plus *pSVK3-GNAS* proline²⁰³, suggesting that the mutant caused an increase in intracellular cAMP levels. These results indicate that, like the Gs α residue arginine²⁰¹, the Gs α amino acid leucine²⁰³ participates in the GTPase turn-off reaction.

PKA plays a central role in the cAMP signalling pathway. In a previous study of a large series of hyperactive thyroid nodules, no mutations were found in the PKA catalytic subunit (43). Correspondingly, in the present TA series, no mutations in the *PRKARIA* gene coding region were found in toxic adenomas. Together, these findings suggest that mutations in PKA are not involved in the development of toxic TAs.

In rat thyroid cells, TSH stimulates cell proliferation via a cAMP-mediated pathway that requires activation of RAS mediated by a mechanism independent of PKA activity (44). *Ras* mutations are observed in both benign and malignant thyroid neoplasias (45, 46) and have also been reported in TA (11). In the present study of TA, no mutations were found in codons 12, 13 or 61 of the *Ras* oncogene, suggesting that if RAS is involved in toxic TA its involvement is minimal.

In the present study, the number of polyclonally derived TAs exceeded those that were monoclonal, with polyclonal tumours exhibiting a higher percentage of mutations. Previous studies had shown a monoclonal origin for nearly all thyroid toxic nodules bearing activating mutations in either the *TSHR* or Gs α protein, as well as for half the number of nodules in which mutations were not found (47). Clonal analysis is affected by the cut-off criterion for clonality, by contamination of samples with connective and/or vascular tissue and by the thyroid patch, a cluster of thyroid progenitor cells with the same pattern of X chromosome inactivation (2). A low cut-off value will increase the number of monoclonal samples thereby decreasing specificity. In the present study, lowering the cut-off value from 3 to 2 (47) would increase the number of monoclonal samples from 6 (26%) to 14 (61%) and would increase the number of mutations in the monoclonal samples from 33.3 to 50%. However, even with a cut-off value of 2, our percentage of polyclonal samples is high. Since a large patch size would bias clonality assays towards monoclonality, the high number of polyclonal nodules detected in our study seems to indicate that DNA samples analyzed were probably a compound mixture of thyrocytes, connective and/or vascular tissue.

Denaturing gradient gel electrophoresis (DGGE) is more sensitive than direct sequencing in detecting *TSHR* gene mutations (48) and DGGE could therefore have been used to increase the likelihood of detecting mutations in thyroid TA samples containing high amounts of connective and/or vascular tissue. However,

direct sequencing can detect amounts of mutated DNA between 12 and 50% of total DNA (48), and it should therefore be sufficiently sensitive to detect mutations in those samples identified as monoclonal using a high cut-off criterion for clonality. Monoclonal TAs without mutations in *TSHR*, *GNAS*, *PRKARIA* or *RAS* genes should have mutations in other as yet unidentified genes.

Although not an aim of the present study, a papillary thyroid carcinoma was incidentally found in 10% of the thyroid surgical specimens studied. The reported incidental finding of thyroid carcinoma among patients undergoing surgery for benign thyroid disease ranges from 3 to 16% (49), including toxic uni- or multinodular goitre (50). Our results are in agreement with those from surgical patients with hyperthyroidism due to toxic multinodular goitre or toxic adenoma where a prevalence of thyroid carcinoma of 7–8% has been reported (51).

In conclusion, nearly 66% of toxic TAs from a large series of patients in Galicia, an iodine-deficient region located in NW Spain, have activating mutations in either the *TSHR* or *GNAS* gene. Based on our study, *PRKARIA* gene and *RAS* genes do not appear to be involved in TA aetiology in that community. Only 2 out of 52 activating *TSHR* gene mutations were previously unreported, suggesting that most *TSHR* mutations have been already reported. A naturally occurring new activating mutation in *GNAS*, L203P, was also found in one TA, showing that residue leucine²⁰³ is important in the regulation of Gs α -subunit intrinsic GTPase activity. The underlying genetic defect(s) remain(s) unknown in one-third of TAs from Galicia.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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