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 (Gsα), 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 Gsα, 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 (RIα), 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 gene 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 ± s.d.) was 53.6 ± 15.4 years (range: 22–78 years). Patients were from six hospitals of the Servicio Galego de Saúde (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 (⁹⁹mTc) 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 ⁹⁹mTc 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 deparaffinized 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 µl 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 µl) 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
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 (Qiagen II Gel Extraction Kit, Qiagen GmbH), fragments were subcloned into a pSVL vector (Promega), transformed into JM109 and verified by direct sequencing. Fragments of 567 bp carrying the mutations were excised from a pSVK3 vector (a gift from Michael A Levine, Children’s Hospital Cleveland Clinic, OH, USA) previously linearized with SalI. 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-α 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, Texas Tech 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.

<table>
<thead>
<tr>
<th>Exon</th>
<th>Forward oligonucleotide</th>
<th>Reverse oligonucleotide</th>
</tr>
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<tbody>
<tr>
<td>PRKAR1A</td>
<td>2</td>
<td>5'-TACAAGCAGTGTGT................TC-3'</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5'-ATGGGAGAGTGACGTGCA-3'</td>
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<td></td>
<td>4–5</td>
<td>5'-GCGGCTGCTAAGCTGGAA-3'</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>5'-GCTTCTAATTTTTACCTCTTCT-3'</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>5'-GTGTTTGGAGTTTATATTAAG-3'</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>5'-ACAGCTTTGAGCTTTGTTT-3'</td>
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<tr>
<td></td>
<td>9</td>
<td>5'-GAGACCAAAATAATACAGACG-3'</td>
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<td></td>
<td>10</td>
<td>5'-TCGTTAGAGTGGTTAAGTGC-3'</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>5'-TCGTTAGAGTGGTTAAGTGC-3'</td>
</tr>
<tr>
<td>KRAS</td>
<td>2</td>
<td>5'-CTGAGGGCAGGCTGAGCAG-3'</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5'-GGGTCCCTGAGCCCTGTC-3'</td>
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<td>2</td>
<td>5'-GCTGCGGAATTAACCTCTGATT-3'</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5'-AGCATGTCATTCCCTGTG-3'</td>
</tr>
</tbody>
</table>

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/ml 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 mU/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 PKAR1A 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; serine 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.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Base substitution</th>
<th>Mutation position</th>
<th>Pathology</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>S281N</td>
<td>AGC→ACC</td>
<td>ECD</td>
<td>MNG-TA</td>
<td>1</td>
</tr>
<tr>
<td>S281T</td>
<td>AGC→ACC</td>
<td>ECD</td>
<td>MNG-TA</td>
<td>1</td>
</tr>
<tr>
<td>S425I</td>
<td>AGT→ATT</td>
<td>1st TMH</td>
<td>TA</td>
<td>2</td>
</tr>
<tr>
<td>G431S</td>
<td>GGC→AGC</td>
<td>1st TMH</td>
<td>TA</td>
<td>1</td>
</tr>
<tr>
<td>G431S</td>
<td>ATG→AGC</td>
<td>1st TMH</td>
<td>MNG-TA</td>
<td>10</td>
</tr>
<tr>
<td>T465V</td>
<td>ATG→GGT</td>
<td>2nd TMH</td>
<td>MNG-TA</td>
<td>2</td>
</tr>
<tr>
<td>I466F</td>
<td>ATT→TTC</td>
<td>1st ICL</td>
<td>MNG-TA</td>
<td>3</td>
</tr>
<tr>
<td>L512R</td>
<td>CTG→CGG</td>
<td>3rd TMH</td>
<td>MNG-TA</td>
<td>2</td>
</tr>
<tr>
<td>L512Q</td>
<td>CTG→CAG</td>
<td>3rd TMH</td>
<td>TA</td>
<td>1</td>
</tr>
<tr>
<td>I568T</td>
<td>ATC→ACC</td>
<td>2nd ECL</td>
<td>MNG-TA</td>
<td>4</td>
</tr>
<tr>
<td>V597F</td>
<td>GTC→TTT</td>
<td>5th TMH</td>
<td>TA</td>
<td>1</td>
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<td>V597L</td>
<td>GTC→CTC</td>
<td>5th TMH</td>
<td>TA</td>
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</tr>
<tr>
<td>D619G</td>
<td>GAT→GTT</td>
<td>3rd ICL</td>
<td>MNG-TA</td>
<td>3</td>
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<tr>
<td>∆619</td>
<td>GAT</td>
<td>3rd ICL</td>
<td>TA</td>
<td>1</td>
</tr>
<tr>
<td>A623V</td>
<td>GCC→GTC</td>
<td>3rd ICL</td>
<td>TA</td>
<td>1</td>
</tr>
<tr>
<td>A623I</td>
<td>GCC→ATC</td>
<td>3rd ICL</td>
<td>TA</td>
<td>1</td>
</tr>
<tr>
<td>*A623F</td>
<td>GCC→TTC</td>
<td>3rd ICL</td>
<td>TA</td>
<td>1</td>
</tr>
<tr>
<td>L629F</td>
<td>TTG→TTT</td>
<td>6th TMH</td>
<td>MNG-TA</td>
<td>2</td>
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<tr>
<td>I630L</td>
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<td>D633E</td>
<td>GAC→GAA</td>
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<td>D633Y</td>
<td>GAC→TAC</td>
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<td>D633H</td>
<td>GAC→CAC</td>
<td>6th TMH</td>
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<tr>
<td>*I635V</td>
<td>ATA→GTA</td>
<td>6th TMH</td>
<td>TA</td>
<td>1</td>
</tr>
<tr>
<td>V656F</td>
<td>GTT→TTC</td>
<td>3rd ECL</td>
<td>TA</td>
<td>1</td>
</tr>
</tbody>
</table>

N, number of mutations; ECD, extracellular domain; TMH, transmembrane domain; ECL, extracellular loop; ICL, intracellular loop; MNG-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 (0.917 C/C homozygous, 0.082 C/A heterozygous)) and amino acid 727 (aspartic acid→glutamic acid, D727E, GA/G (0.919 C/C, 0.081 C/G)).

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, C/CC/T (0.940 C/C homozygous, 0.060 C/T heterozygous), glycine 185, AT/C/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 PRKAR1A 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...
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 Gαs 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). Gαs arginine201 is the site at which cholera toxin catalyses ADP-ribosylation inhibiting Gαs subunit intrinsic GTPase activity. As a result of reduced GTPase activity, Gαs remains in its active GTP-bound form causing an unregulated activation of adenyl cyclase (41). Replacement of arginine201 by histidine decreases intrinsic GTPase activity and leads to activation of adenyl cyclase (41). Gαs residue glutamine227 belongs to the protein guanine nucleotide binding domain (42). Replacement of glutamine227 by glutamic 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 adenyl cyclase (42). No activating GNAS mutations have previously been reported at Gαs residue leucine203, located near arginine201. A naturally occurring substitution of
leucine\textsuperscript{203} 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 proline\textsuperscript{203}, suggesting that the mutant caused an increase in intracellular cAMP levels. These results indicate that, like the Gs\textsubscript{z} residue arginine\textsuperscript{211}, the Gs\textsubscript{z} amino acid leucine\textsuperscript{203} 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 PRKAR1A 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\textsubscript{z} 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 affected by the cut-off criterion for clonality, by toxic TA its involvement is minimal.

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, PRKAR1A 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 leucine\textsuperscript{203} is important in the regulation of Gs\textsubscript{z}-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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