Autonomously functioning thyroid nodules in a former iodine-deficient area commonly harbor gain-of-function mutations in the thyrotropin signaling pathway

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Abstract

Background: Somatic activating mutations of the thyrotropin (thyroid-stimulating hormone (TSH)) receptor (TSHR) and Gαs protein have been detected in solitary toxic adenomas and toxic multinodular goiters, but their role in the pathogenesis of autonomous nodules is debated. The frequency of mutations is highly variable among populations and is inversely proportional to iodine intake.

Design and patients: We screened 28 clinically and histologically heterogeneous autonomous nodules from 24 Greek patients for the presence of TSHR and Gαs mutations.

Results: By direct sequencing of genomic DNA, we detected 11 somatic heterozygous gain-of-function mutations in TSHR and one in Gαs. Forty-three percent (12 of 28) of all nodules and 57% (four of seven) of solitary toxic adenomas harbored an activating mutation. Typical adenomas and hyperplastic nodules did not differ in mutation frequency. Substitutions I568T and T632I were detected in both histological types of nodules.

Conclusions: Our findings indicate that activating somatic mutations in the TSH signaling pathway are frequent in autonomous nodules in Greece. This may be due to earlier exposure of the population to iodine deficiency, which was corrected in Greece only over the past two decades. Gain-of-function mutations are shared by nodules with varying histological and clinical presentations. Thus, they may represent a common molecular mechanism underlying the pathogenesis of non-autoimmune thyroid autonomy.

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Introduction

The proliferation and differentiation of thyroid cells are regulated mainly by thyrotropin (thyroid-stimulating hormone (TSH)) (1), which acts on thyrocytes by binding its cognate receptor (TSH receptor (TSHR)) at the thyroid cell plasma membrane. TSHR has the typical structure of G protein-coupled receptors: it consists of seven transmembrane segments interconnected by three extra- and three intracellular loops (2). The large extracellular N-terminus largely confers TSH-binding specificity, while the intracellular C-terminus participates in downstream signal transduction. Hormone binding to the receptor activates adenylate cyclase through the Gαs protein, and thus increases the intracellular levels of cAMP (2, 3), which is the prime second messenger of TSH in the thyroid (1). At higher hormone concentrations, the phospholipase C pathway is also activated via Gαq (4).

The fundamental role of TSH in thyroid cell growth and function led to the hypothesis that somatic gain-of-function mutations of the proteins forming the hormone signaling pathway would cause the clonal expansion of the affected cell (5). The subsequent generation of a clone of autonomously growing and functioning thyrocytes would correspond to the clinical phenotype of a hyperfunctioning thyroid nodule (6). Indeed, activating mutations were found in solitary toxic adenomas, initially in the Gαs protein (7) and later in the TSHR (8). These mutations are somatic, heterozygous and confined to the autonomous tissue. They induce the activation of the cAMP and/or phospholipase C pathway in a constitutive (TSH-independent) manner (9). Gαs mutations affect only two amino acid residues; substitutions at these critical sites impair GTP hydrolysis, thus maintaining Gαs in the active state (7). On the other hand, TSHR mutations reside in exons 9 and 10 of the receptor.
gene, that encode the intracellular, transmembrane and proximal extracellular TSHR fragments; thus, mutations are spread across the carboxy-half of the receptor (9). TSHR mutations are usually amino acid substitutions and, less commonly, small deletions. They induce receptor constitutive activity by interrupting the intramolecular electrostatic bonds that constrain the TSHR in a minimally active conformation (9, 10).

Apart from solitary toxic adenomas, mutations activating the TSHR and $G_{\alpha S}$ were subsequently found in autonomous nodules within toxic or autonomous multinodular goiters (11, 12). Autonomous nodules are histologically classified either as typical follicular adenomas, that are relatively homogeneous and surrounded by a complete capsule, or as hyperplastic adenomas, that are relatively homogeneous and surrounded by a complete capsule, or as hyperplastic adenomas, that are more heterogeneous and not encapsulated (13, 14). Activating $G_{\alpha S}$ and TSHR mutations have been detected in all these clinical and histological subtypes of autonomous nodules, but have never been found in non-functioning nodules (15, 16). Thus, although not present in all autonomous nodules, gain-of-function mutations emerge as a common molecular mechanism underlying the pathogenesis of non-autoimmune thyroid autonomy (3, 9).

Studies screening autonomous nodules report widely varying frequencies of TSHR and $G_{\alpha S}$ activating mutations among different populations (17). In a series of toxic adenomas, mutation frequencies range from 0 to 82% for TSHR and from 3 to 75% for $G_{\alpha S}$. These extreme variations are partly attributed to methodological problems (18, 19). Additionally, genetic and cytogenetic factors, such as dysregulated expression and function of G proteins, may act in concert with the natural heterogeneity of thyroid cells and/or environmental agents to beget hyperfunctioning nodules lacking TSHR and $G_{\alpha S}$ mutations, or to modify the clinical presentation of gain-of-function mutations (20). A differential incidence of such factors among populations could account for the conflicting frequencies of activating mutations. Iodine deficiency has been proposed as a plausible culprit, since TSHR and $G_{\alpha S}$ mutation frequency is inversely related to iodine intake (20). Iodine deficiency was endemic in post-Second World War Greece (21), but has been significantly rectified over the past 20 years and goiter prevalence has decreased accordingly (22–24).

The aim of our study was to assess the prevalence of TSHR and $G_{\alpha S}$ gain-of-function mutations in hyperfunctioning nodules from toxic or autonomous uni- or multinodular goiters in Greek subjects.

**Materials and methods**

**Subjects**

The study included 24 patients from Southwestern Greece who were subjected to thyroidectomy due to autonomous or toxic uni- or multinodular goiter (21 women and three men, median age 45 years, range 21–70). Before thyroidectomy, patients were clinically evaluated in detail and underwent thyroid ultrasound scans and $^{99m}$Tc scintiscans. Serum thyroxine, free thyroxine, triiodothyronine, free triiodothyronine and TSH concentrations and thyroid autoantibodies were measured. Eighteen out of 24 patients were hyperthyroid at diagnosis, with high values of serum thyroid hormones and suppressed TSH. Seven of these harbored solitary hot nodules and 11 had toxic multinodular goiters. The remaining six patients had normal thyroid hormone levels and low serum TSH values; subclinical hyperthyroidism was due to autonomous multinodular goiter. Thyroid autoantibodies (anti-thyroid peroxidase antibodies and anti-thyroglobulin antibodies) were negative in all patients. $^{99m}$Tc scans revealed that 21 patients harbored a solitary hot nodule, two subjects had two hot nodules each, and the remaining patient carried three autonomous nodules. Thus, the total number of autonomous nodules/areas rose to 28.

The study was in accordance with the principles of the Declaration of Helsinki and was approved by the University of Patras Research Committee. Patients gave their informed consent before the operation, which was decided in each case by the attending physician irrespectively of the conduct of this study.

All subjects were natives of Southwestern Greece. Fifty percent of them were residents of mountainous villages and 50% city dwellers.

**Methods**

Immediately after thyroidectomy, tissue samples were taken from the autonomous nodules and surrounding normal tissue. Samples were carefully excised matching the scintiscan and ultrasound patterns with the whole gland laid in its proper anatomical orientation. Tissue specimens were shock-frozen in liquid nitrogen. Peripheral blood was collected in EDTA and stored at $-20^\circ$C. Nodules were classified as typical adenomas or hyperplastic nodules according to conventional pathological criteria (14). Of 28 hyperfunctioning areas, 11 were characterized as follicular adenomas and 17 as hyperplastic nodules. Lymphocytic infiltration or other stigmata of autoimmunity were not detected in any sample.

Frozen tissue samples were pulverized, cells were lysed and genomic DNA extracted by the standard phenol/chloroform procedure. Genomic DNA was extracted from blood samples with Nucleospin Blood QuickPure kit (Macherey-Nagel, Düren, Germany). TSHR exons 9 and 10 and $G_{\alpha S}$ exons 7–10, where activating mutations have been found in hot nodules, were amplified by PCR (25).

Oligonucleotide primers (Table 1) were synthesized by MWG-Biotech AG, Ebersberg, Germany. TSHR exon 9, two overlapping fragments encompassing
TSHR exon 10 and a segment spanning exons Ga as exons 7–10 were amplified by PCR; 100 ng genomic DNA were used as template in a PTC-200 thermocycler (MJ Research Inc., Waltham, MA, USA). PCR was performed in a total volume of 50 μl, using 10 pmol each appropriate primer, 200 μmol/l each dNTP, 1.5 mmol/l MgCl₂, 2.5 U Gibco Brl Taq DNA polymerase (ANTI-SEL; Thessaloniki, Greece) and 10 μl reaction buffer containing 200 mmol/l Tris–HCl (pH 8.4) and 500 mmol/l KCl. After an initial denaturing at 95 °C for 5 min, reactions were subjected to 30 cycles of 30 s denaturing at 95 °C, annealing at the appropriate temperature and extension at 72 °C, followed by a final 6 min extension at 72 °C.

PCR products were purified with Nucleospin Extract kit (Macherey-Nagel). At least two different purified PCR amplicons were sequenced on both sense and anti-sense strands. Sequencing reactions were performed using ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Weiterstadt, Germany) with the PCR primers as sequencing primers and run on an ABI 373 DNA Sequencer (Applied Biosystems, Foster City, CA, USA).

Comparison of mutation frequency between typical adenomas and hyperplastic nodules was performed by chi-square cross-tabulation (SPSS 9.0 for Windows; SPSS Inc., Chicago, IL, USA).

**Results**

In a series of 28 autonomous thyroid nodules, we found 12 somatic point mutations, 11 in the TSHR gene and one in the Ga as gene (Table 2). The mutations were confined in the autonomous tissue and not detected in neighboring normal thyroid parenchyma or peripheral blood leukocytes. All mutations were heterozygous, appearing as double peaks in chromatograms, and coded for single amino acid substitutions (missense mutations). All except one have been identified in previous studies and their functional characteristics have been studied in transient transfection experiments, where they were shown to constitutively activate the TSHR or Ga as (3). The substitution of alanine with asparagine at codon 593 (A593N) in the fifth transmembrane helix, which resided on the same TSHR allele, with the germline polymorphism D727E has not been described before and we have recently described its functional characteristics (26). All TSHR mutations resided in exon 10 and each mutation was found in a different patient. The I568T substitution was detected in two patients, in a typical adenoma in the first and in a hyperplastic nodule in the other; the same was observed for mutation T632I.

The 24 patients studied harbored 28 autonomous nodules/areas, of which 12 were found to bear a

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**Table 1** Oligonucleotide primers and conditions for amplification of analyzed genomic DNA sequences.

<table>
<thead>
<tr>
<th>PCR Gene</th>
<th>Oligonucleotide primers</th>
<th>Annealing temperature (°C)</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 TSHR, exon 9</td>
<td>F: 5'-TCA TCT CCC AAT TAA CCT CAG G-3'</td>
<td>64</td>
<td>408</td>
</tr>
<tr>
<td>2 TSHR, exon 10</td>
<td>R: 5'-GCT TCC AAT TTC CTC TCC AC-3'</td>
<td>66</td>
<td>868</td>
</tr>
<tr>
<td>3 TSHR, exon 10</td>
<td>F: 5'-ACT GTC TCT CTG GCA GAT GC-3'</td>
<td>66</td>
<td>875</td>
</tr>
<tr>
<td>4 Ga as, exon 7–10</td>
<td>R: 5'-GTC TCA TGG TAT TGG GAT GC-3'</td>
<td>62</td>
<td>610</td>
</tr>
</tbody>
</table>

**Table 2** Somatic TSHR and Ga as gain-of-function mutations detected in the hyperfunctioning thyroid nodules studied.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Residue substitution</th>
<th>TSHR region</th>
<th>Gene</th>
<th>Nodule histology</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 atg → acg</td>
<td>M453T</td>
<td>TM2</td>
<td>TSHR, exon 10</td>
<td>Hyperplastic</td>
<td>Toxic multinodular goiter</td>
</tr>
<tr>
<td>2 atc → ttc</td>
<td>I486F</td>
<td>EL1</td>
<td>TSHR, exon 10</td>
<td>Adenoma</td>
<td>Toxic multinodular goiter</td>
</tr>
<tr>
<td>3 ctg → cag</td>
<td>L512Q</td>
<td>TM3</td>
<td>TSHR, exon 10</td>
<td>Hyperplastic</td>
<td>Autonomous multinodular goiter</td>
</tr>
<tr>
<td>4 atc → acc</td>
<td>I568T</td>
<td>EL2</td>
<td>TSHR, exon 10</td>
<td>Hyperplastic</td>
<td>Autonomous multinodular goiter</td>
</tr>
<tr>
<td>5 atc → acc</td>
<td>I568T</td>
<td>EL2</td>
<td>TSHR, exon 10</td>
<td>Adenoma</td>
<td>Solitary toxic adenoma</td>
</tr>
<tr>
<td>6 gcc → aac</td>
<td>A593N</td>
<td>TM5</td>
<td>TSHR, exon 10</td>
<td>Adenoma</td>
<td>Solitary toxic adenoma</td>
</tr>
<tr>
<td>7 tat → aat</td>
<td>Y601N</td>
<td>TM5</td>
<td>TSHR, exon 10</td>
<td>Hyperplastic</td>
<td>Toxic multinodular goiter</td>
</tr>
<tr>
<td>8 tgc → ttc</td>
<td>L629F</td>
<td>TM6</td>
<td>TSHR, exon 10</td>
<td>Hyperplastic</td>
<td>Toxic multinodular goiter</td>
</tr>
<tr>
<td>9 acc → atc</td>
<td>T632I</td>
<td>TM6</td>
<td>TSHR, exon 10</td>
<td>Hyperplastic</td>
<td>Solitary toxic adenoma</td>
</tr>
<tr>
<td>10 acc → atc</td>
<td>T632I</td>
<td>TM6</td>
<td>TSHR, exon 10</td>
<td>Hyperplastic</td>
<td>Toxic multinodular goiter</td>
</tr>
<tr>
<td>11 gac → gag</td>
<td>D633E</td>
<td>TM6</td>
<td>TSHR, exon 10</td>
<td>Hyperplastic</td>
<td>Solitary toxic adenoma</td>
</tr>
<tr>
<td>12 cgt → cat</td>
<td>R201H</td>
<td>—</td>
<td>Ga as, exon 8</td>
<td>Hyperplastic</td>
<td>Solitary toxic adenoma</td>
</tr>
</tbody>
</table>

*TM, transmembrane domain; EL, extracellular loop.*
mutation. Thus, one out of two patients had a somatic activating mutation, whereas the percentage of mutations over nodules was 43% (12 of 28) and 39% (11 of 28) with regards the TSHR and 4% (1 of 28) the G\textsubscript{\alpha}s protein. Eleven hot areas were histologically identified as follicular adenomas and 17 as hyperplastic nodules. Mutations were found in six of 11 adenomas (55%) and six of 17 hyperplastic nodules (35%). The frequency of mutations did not differ between these two histological subtypes of autonomous nodules (likelihood ratio = 0.626, P = 0.47). Out of seven nodules that fulfilled the clinical and histological criteria of solitary toxic adenoma, four harbored a mutation; this frequency of 57% is analyzed into 43% (three of seven) for TSHR and 14% (one of seven) for G\textsubscript{\alpha}s.

Apart from somatic mutations of TSHR and G\textsubscript{\alpha}s, genetic analysis also demonstrated some germline variations in the patients of the study. The germline nature of these nucleotide substitutions was documented by their presence in the peripheral leukocytes’ DNA, in addition to hyperfunctioning and normal thyroid tissue. Three of the 12 patients with a somatic mutation and two of the 12 without, carried a heterozygous c → g substitution at TSHR cDNA position 2181. This constitutes a TSHR polymorphism resulting from the substitution of aspartate by glutamate at amino acid position 727 (D727E) (27). Additionally, in a patient without any somatic mutation, a heterozygous germline c → g substitution was found in G\textsubscript{\alpha}s intron 7, at a nucleotide 51 bp downstream from exon 7 and 81 bp upstream from exon 8.

No difference was detected in mutations frequencies between city dwellers and villagers.

The status of iodine intake and its relation to thyroid function has been previously evaluated in 827 residents of Southwestern Greece aged 15–80 years (23). Urinary iodine excretion (UIE) > 10 μg/dl was present in 54% of the entire population. According to these values, Southwestern Greece is no longer an iodine-deficient area. However, in contrast to city dwellers in whom only 35% had UIE < 10 μg/dl, among villagers 54% had UIE < 10 μg/dl. 23% had UIE < 5 μg/dl and 7% had UIE < 2 μg/dl, suggesting the persistence of mild iodine deficiency among mountainous villagers. The goiter prevalence by palpation was 18% and it was similarly distributed between villagers and city dwellers. Serum TSH had a tendency to be lower in subjects with relatively low iodine intake and higher in groups with either very low or normal–high iodine intake (23).

Discussion

Somatic activating mutations in the TSH signal transduction pathway have been proposed as a possible common pathogenetic mechanism underlying non-autoimmune thyroid autonomy (3, 9). This was judged from the fundamental role of TSH for thyroid cell growth and function (1) and has been substantiated by the detection of TSHR (8) and G\textsubscript{\alpha}s (7) gain-of-function mutations in autonomous nodules. Solitary toxic adenomas and hot nodules within autonomous or toxic multinodular goiters harbor common activating mutations (11, 12). The varying prevalence of mutations makes their importance in the pathogenesis of hot nodules questionable (20). Our study reports a total mutation frequency of 43%; 39% for TSHR and 4% for G\textsubscript{\alpha}s. These mutant receptors were shown to constitutively activate the TSHR and downstream cAMP and/or phospholipase C pathway (3). The G\textsubscript{\alpha}s mutation R201H was shown to impair GTP hydrolysis, thus prohibiting G\textsubscript{\alpha}s inactivation (7).

The divergent frequencies of activating mutations among populations may be partly due to the different methods employed in various studies (18, 19). When sample selection is ideal, the percentage of the heterozygous mutant allele in the sample is 50%. However, very frequently, samples are contaminated with blood, fibroblasts and adjacent normal thyroid cells, especially when the nodule has undergone partial necrosis, cystic degeneration, calcification or fibrosis. Thus, the percentage of mutant allele in the sample decreases below 50% and possibly below the sensitivity threshold of the mutation detection method. Direct sequencing and denaturing gradient gel electrophoresis (DGGE) are highly sensitive detection methods (28). Single-strand conformation polymorphism (SSCP) has low sensitivity, while restriction fragment length polymorphism (RFLP) cannot identify novel mutations (18). Therefore, the low mutation frequencies reported by studies employing these latter methods may be unreliable. Studies that did not examine TSHR and G\textsubscript{\alpha}s in the same series of nodules must also be interpreted with caution. In our study, genetic analysis of meticulously selected samples was carried out by automated sequencing. We found activating mutations in 57% of solitary toxic adenomas. This finding is in agreement with the results of Trulzsch et al. (28), who studied a large series of toxic adenomas by DGGE and direct sequencing and found mutations in 60% of cases.

Underlying differences of the respective patient populations (20) may also account for the reported variations in mutation frequency. Activating mutations in the TSH signal transduction pathway are related to iodine intake: they are common in relatively iodine-deficient countries like Italy (15) but rare in iodine-sufficient countries like Japan (29). In the latter, a recent study focusing on solitary adenomas, instead of multinodular goiters, found an incidence rate (70%) similar to that found in European patients (30). Iodine deficiency increases serum TSH levels as well as the sensitivity of the thyroid gland to the stimulatory effects of TSH (31). This in turn stimulates mitotic activity in the thyroid, thus increasing the probability of a mutation occurring in a replicating cell (24). Moreover, iodine deficiency favors an intracellular environment.

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rich in free oxygen radicals that promote mutagenesis. Alternatively, TSH-induced thyroid stimulation may promote the clonal selection of mutated cells, since mutant receptors retain the potential for further stimulation by TSH (26). Iodine deficiency was endemic in post-Second World War Greece, but has been corrected over the last 20 years by increased use of iodized salt and improved socioeconomic conditions (21). As a result, the prevalence of goiter has substantially decreased (22). However, iodine deficiency still persists in some mountainous regions, including areas of Southwestern Greece (23). The patients in this report were all residents of Southwestern Greece and all were older than 20 years of age. The majority of them harbored multinodular goiters instead of solitary adenomas. Thus, they have all been exposed to an iodine-deficient environment, at least early in life. This may explain the observed mutation incidence of 43%, which is intermediate between the reported extreme ranges.

The distribution of mutated residues on the TSHR protein is of particular importance. Four mutations were found in the sixth transmembrane segment (TM6), three in the first two extracellular loops and the remaining mutations in TM2, TM3 and TM5. This distribution is in agreement with previous data that highlight these TSHR areas as hot-spots of activating mutations (3). TSHR mutation database: http://www.uni-leipzig.del-inneze/tsh/). These findings, in association with the fact that the wild-type TSHR displays a small but substantial constitutive signaling activity (8), support the current model for TSHR structure-function relationship (32–35). According to this model, the TSHR exists in equilibrium between active and inactive conformations, and TSH tilts this balance by stabilizing the active receptor configuration. Non-covalent bonds between the side-chains of critical amino acid residues maintain the receptor in its most inactive conformation. Through such bonds, the extracellular loops are interconnected with the proximal extracellular fragment of the receptor and the transmembrane domains are tightly packaged together (32). The signaling TSHR conformation is characterized by a loosening of these bonds and a repositioning of the transmembrane domains, so that a movement of TM6 towards the cytoplasm enables the third intracellular loop to make contact with and activate $G_{\text{ras}}$ (33). Activating mutations affect residues critical for the formation of intramolecular bonds and thus enable the receptor to adopt configurations that display constitutive activity (34). The distribution of activating mutations in extracellular loops 1 and 2 and TMs 2, 3, 5 and 6 observed in our study is fully compatible with the latter view and adds further credit to the above model of TSHR structure-function relationship.

In conclusion, we found somatic mutations constitutively activating TSHR and $G_{\text{ras}}$ in a group of clinically and histologically heterogeneous autonomous nodules. The observed mutation incidence among patients earlier exposed to an iodine-deficient environment is intermediate between the reported extreme ranges. The frequency of mutations was similar in typical follicular adenomas and hyperplastic nodules. Two activating mutations, I568T and T632I, were detected in the sixth transmembrane segment (TM6), three in the first two extracellular loops and the remaining mutations in TM2, TM3 and TM5. This distribution is in agreement with previous data that highlight these TSHR areas as hot-spots of activating mutations (3). TSHR mutation database: http://www.uni-leipzig.del-inneze/tsh/). These findings, in association with the fact that the wild-type TSHR displays a small but substantial constitutive signaling activity (8), support the current model for TSHR structure-function relationship (32–35). According to this model, the TSHR exists in equilibrium between active and inactive conformations, and TSH tilts this balance by stabilizing the active receptor configuration. Non-covalent bonds between the side-chains of critical amino acid residues maintain the receptor in its most inactive conformation. Through such bonds, the extracellular loops are interconnected with the proximal extracellular fragment of the receptor and the transmembrane domains are tightly packaged together (32). The signaling TSHR conformation is characterized by a loosening of these bonds and a repositioning of the transmembrane domains, so that a movement of TM6 towards the cytoplasm enables the third intracellular loop to make contact with and activate $G_{\text{ras}}$ (33). Activating mutations affect residues critical for the formation of intramolecular bonds and thus enable the receptor to adopt configurations that display constitutive activity (34). The distribution of activating mutations in extracellular loops 1 and 2 and TMs 2, 3, 5 and 6 observed in our study is fully compatible with the latter view and adds further credit to the above model of TSHR structure-function relationship.

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