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Toxic thyroid adenoma: absence of DNA mutations of the TSH receptor and Gsα

C Pinducciu, G Borgonovo[1], A Arezzo[1], G C Torre[1], G Giordano and R Cordera

Department of Endocrinology and Metabolism and [1]First Clinica Chirurgica, University of Genova, Genova, Italy

(Correspondence should be addressed to R Cordera who is now at DiSEM, University of Genova, Viale Benedetto XV 6, 16132 Genova, Italy)

Abstract

DNA point mutations of the TSH receptor and of the α subunit of the stimulatory GTP-binding protein (Gsα) have been suggested as major causes of hyperfunctioning thyroid adenomas. However, significant differences in the prevalence of these mutations (from 0·3 to 84%) have been found in different populations. The present study was designed to evaluate further the presence of mutations in discrete fragments of cDNA encoding critical regions of the TSH receptor and of the Gsα involved in signal transduction and cAMP production. Genomic DNA extracted from 15 thyroid adenomas and surrounding quiescent thyroid tissues was used as a template to amplify four DNA fragments of TSH receptor and one DNA fragment of Gsα. TSH receptor and Gsα DNAs were analyzed by a number of techniques. We did not detect any mutations (new or previously described) in our patients. These results confirm that the causes of solitary toxic adenomas are protean, and only some of them may be somatic DNA point mutations. Since the clinical features of solitary toxic adenoma are homogeneous, it could be important to establish the specific molecular defect underlying each case, in order to follow up the patients and to assess their clinical evolution.

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Introduction

Point mutations of the DNA encoding the receptor for thyrotropin (TSH-R) and the α subunit of the stimulatory GTP-binding proteins (Gsα) have been suggested as a molecular cause of toxic thyroid adenomas (1–7). These mutations act through the constitutive (independent of TSH) activation of the specific receptor and/or the downstream G-protein pathway, leading to an increase in the intracellular content of cAMP. This in turn promotes thyroid cell hyperfunction and growth (8, 9).

These mutations are somatic, since they have been found only in the affected tissue and result from unknown acquired events (1), in contrast with germinal TSH mutations found in familial cases of hyperthyroidism (10).

As predicted by structure/function studies, most of the mutations causing toxic adenomas have been found in the third cytoplasmic loop and the sixth transmembrane segment of the TSH-R and in the C-terminus of Gsα, which are critical in receptor interactions ('hot spot' sequences) (9, 11–15). However, two extracellular mutations causing toxic thyroid adenomas have been reported (16).

The prevalence of these mutations varies from 0.3 to 84% (6, 17). These two extremes have been described in a Japanese and a Caucasian population respectively, suggesting that ethnic factors and iodine intake may be important in the aetiology of toxic thyroid adenomas.

To assess further the role of DNA point mutations in these TSH-R and Gsα 'hot spots' in the pathogenesis of thyroid solitary adenomas, we studied the prevalence of DNA point mutations of TSH-R and Gsα in a selected population of patients undergoing elective partial thyroidectomy for solitary toxic adenoma.

Materials and methods

Subjects

Human thyroid tissues from 15 patients with hyperfunctioning adenomas were obtained at the time of surgery and immediately frozen in liquid nitrogen until used for DNA studies. In all patients, a single hot nodule visualized by ^{125}I scan, with suppression of the surrounding thyroid tissue, undetectable TSH and elevated serum free thyroxine and tri-iodothyronine concentrations, was present (Table 1). Antibodies against TSH-R were absent as well as anti-peroxidase and anti-thyroglobulin antibodies. Histologic examination confirmed that all nodules corresponded to encapsulated hyperfunctioning follicular tissue.

Table 1 Features of patients with toxic thyroid adenomas.

Patients	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Age (years)	51	59	57	77	47	67	37	54	53	37	59	79	64	60	52
Sex	F	F	F	M	F	F	F	M	F	F	F	F	M	F	M
TSH (μU/ml)	0.02	<0	<0	0.04	<0	0	UD	<0	<0	UD	UD	UD	UD	0.03	UD

UD, undetectable.

DNA extraction

DNA was extracted from hyperfunctioning nodular tissue and surrounding normal thyroid tissue according to standard protocols (18). After homogenization the tissue samples were incubated at 50 °C for 16 h in the presence of 100 mg/ml proteinase K (Boehringer, Mannheim, Germany) in buffer containing 1 M NaCl, 1 M Tris–HCl, 0.5 M EDTA and 0.5% SDS, pH 8. DNA was purified by phenol, precipitated in ethanol and resuspended in 500 μl TRIS-EDTA (TE). After quantification by measuring absorption at 260 nm, DNA was stored at −20 °C until used.

PCR amplification

Four fragments of TSH-R DNA were amplified by PCR. For fragment A (nucleotides 276–396) the following primers were used: 5′-TTCTTACCCAAGCCACTGC and 5′-ACCATGTCTTCACTGTCCCC; for fragment B (nucleotides 397–493), the following primers were used: 5′-TGGGGACAGTGAAGACATGG and 5′-GAAGAAACCAGCCGTGTTGC; for fragment C (nucleotides 474–582), the following primers were used: 5′-ACCTCTACACTCACTCTGAG and 5′-ATATGCCAGAGCAAGAGGGG; for fragment D (nucleotides 583–679), the following primers were used: 5′-ATTGTTTTTGTTCTGACGC and 5′-GCATAGAGGAATGGATTGGC.

One fragment of Gsα DNA, nucleotides 196–239, containing exons 8 and 9 was amplified by using the following primers: 5′-CCTCAATTTTGTTTCAGGA and 5′-TCTTTACGAACAGCCAAGCC.

PCRs were performed in a final volume of 100 μl with 1 μg DNA, 1.5 mM dideoxyNTPs, 1 U *Taq* polymerase (Boehringer) and 150 mM concentration of each primer in buffer containing 50 mM KCl, 10 mM Tris–HCl, pH 8.3, 2 mM $MgCl_2$, and 0.01% gelatine. After denaturation at 95 °C for 2 min, 30 cycles (95 °C for 30 s, 42 °C for 30 s, 72 °C for 1 min) were followed by a final elongation step of 6 min at 72 °C.

KpnI digestion

Amplified fragment B of TSH-R was precipitated in ethanol and resuspended in 10 μl TE. KpnI digestion was performed in a final volume of 20 μl of appropriate buffer (Boehringer). After incubation at 37 °C for 1 h, the sample was loaded on a 2% agarose gel and electrophoresed.

Sequencing

The PCR products were purified by agarose gel chromatography and sequenced in both sense and antisense directions by dideoxynucleotide chain termination using the Thermo sequenase radiolabeled terminator cycle sequencing kit (Amersham International, Amersham, Bucks, UK). A 2 μl volume of each reaction mixture was loaded on a 6% denaturing gel and electrophoresed for 2–12 h. The gel was autoradiographed overnight at −80 °C.

Allele-specific hybridization

A 20 ng sample of the amplified fragment of Gsα from 12 patients was transferred to Hybond-N membranes (Amersham) and fixed for 2 h at 80 °C.

The blots were prehybridized and hybridized in solution containing 5×SSPE, 50 mg salmon sperm DNA, 5×Denhardt's solution and 0.5% SDS. Prehybridization was performed for 1 h at 65 °C and hybridization for 12 h at 65 °C.

The oligonucleotides were end-labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase (New England Nuclear, Boston, MA, USA). The blots were washed once at room temperature and twice at 65 °C with decreasing concentrations of SSPE before autoradiography. The allele-specific oligonucleotides used in this experiment were: wild-type (TTTCTTGACATTCACCCCAGT) and mutant (TTTCTTGACAC*TCACCCCAG).

Results

The DNA extracted from the 15 thyroid adenomas and the quiescent surrounding thyroid tissues was used as a template to amplify four fragments of TSH-R cDNA, encompassing almost the entire exon 10, and one fragment of Gsα cDNA, encoding nucleotides 196–239 of DNA corresponding to exons 8 and 9. These DNA regions were chosen because all mutations have so far been found in these fragments. Tissue samples were classified as autonomous hyperfunctioning adenomas according to clinical and histopathological criteria.

The samples were first analyzed to look for the presence of the mutation involving the replacement of Asp-619 with Gly (1). This mutation creates a KpnI restriction site, making it easily detectable by enzyme digestion analysis: the appearance of the DNA fragments is indicative of the presence of this mutation.

Figure 1 Direct nucleotide sequencing of one sample of PCR-amplified DNA of TSH-R. No mutations were found in the tissues from any of the 15 patients. g indicates base change.

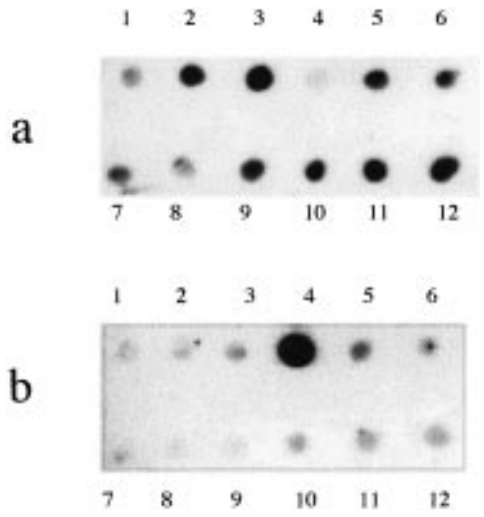

Figure 2 Allele-specific hybridization of DNA obtained by PCR amplification of Gsα. The numbers indicate the patients. Hybridization with wild-type oligonucleotide (a) and mutant oligonucleotide (b) is shown.

None of our patients showed the digestion pattern characteristic of the mutation.

The DNA samples were then analyzed by direct sequencing in order to detect other point mutations. The sequences of the amplified DNA of TSH-R from both the adenoma and the surrounding tissue were compared with that of the wild-type TSH-R gene sequence. Again, no mutations were found in the tissues from the 15 patients (Fig. 1). In contrast, the sequence of Gsα revealed, in one patient, the presence of one heterozygotic mutation, C to T at position 50 in the intron flanked by exons 8 and 9. This mutation was present in both the adenoma and the adjacent thyroid tissue. Its involvement in the pathogenesis of the solitary toxic thyroid adenoma is therefore unlikely.

To confirm the presence of this mutation, a fragment of Gsα was amplified from DNA extracted from peripheral leukocytes and several samples of thyroid tissue and analyzed by allele-specific hybridization as described in Materials and methods. Amplified DNA was dotted on to a nitrocellulose filter and hybridized with ^{32}P-end-labeled oligonucleotide reproducing the mutation and the ^{32}P-end-labeled oligonucleotide reproducing the wild-type sequence. Fig. 2 shows the results of allele-specific hybridization, which confirm the presence of the mutation in the tissue samples of the patient.

Discussion

In our series of toxic adenomas, the mechanisms of mutational activation are not present and other cellular alterations must be responsible for the hyperfunctioning.

This finding is not unexpected, since the prevalence of TSH-R mutations in toxic thyroid adenomas varies in different studies. Studies in Caucasian populations demonstrated that TSH-R point mutations are present in the majority (about 80%) of hyperfunctioning thyroid adenomas (6). In contrast, a recent report has shown that the prevalence of these mutations in a Japanese population is extremely low (17). It has been hypothesized that the different iodine intakes in European and Japanese populations could explain these contrasting results (17, 19). This hypothesis does not explain our findings, since iodine intake in our patients was similar to that in other European populations analyzed. Regardless of the possible explanation, it is evident that several toxic adenomas are not secondary to TSH-R DNA point mutations. It may be possible that other genes encoding proteins involved in the TSH-R pathway are mutationally activated in some adenomas.

The biology of mutations of the G-protein-coupled receptor, on the other hand, may be quite protean. Mutations in Gsα DNA have also been reported in non-functioning pituitary adenomas (20), suggesting a potential role for cAMP in cell growth rather than in cell hyperfunction.

Another possible explanation for the apparent discrepancy between our findings and those of others is that clinically homogeneous toxic solitary thyroid adenomas represent heterogeneous diseases; indeed, it is known that some but not all solitary nodules can be followed by the late recurrence of other thyroid adenomas (21). It is therefore possible that the diagnosis of 'solitary toxic adenomas' will be challenged in the follow-up of patients.

The rationale to study only the hot spot region of DNA is based on the structure/function relationship of TSH-R (9, 11–15). It is unlikely therefore that mutations in other regions close to the hot spot are responsible for the hyperfunctioning.

The conclusions suggested by the present study should be interpreted with caution, since our population sample is relatively small and only the hot spot region has been sequenced. However, this study, in agreement with previous ones, points out that the molecular biology of toxic adenomas cannot be explained entirely by point mutations and that other cellular mechanisms must play a role in their development.

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