

CLINICAL STUDY

Similar prevalence of somatic TSH receptor and Gs α mutations in toxic thyroid nodules in geographical regions with different iodine supply in Turkey

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Abstract

Objective: Differences in iodine intake could account for the variable prevalences reported for somatic TSH receptor (TSHR) mutations in toxic thyroid nodules (TTNs). However, this question has not been settled, since no study has yet determined the TSHR mutation prevalence in regions with different iodine supplies in the same population using the same methodology. Therefore, we studied the prevalence of somatic TSHR mutations in TTNs from patients living in iodine-deficient or -sufficient regions in Turkey.

Design and methods: We screened 74 TTNs for somatic TSHR mutations. Exons 9 and 10 of the TSHR and 7 and 8 of the Gs α were screened by denaturing gradient gel electrophoresis. Determination of X-chromosome inactivation was used for clonality analysis.

Results: TSHR mutations were identified in 52 (70.2%) of 74 TTNs. A Gs α mutation was identified in one TTN. Three new TSHR mutations were detected (A627V, I640K, I486N). No significant difference between frequencies of TSHR mutations in iodine deficient/sufficient regions was found. The frequency of non-random X-chromosome inactivation was similar in iodine-sufficient or -deficient regions and in TSHR mutation positive or negative hot nodules.

Conclusions: These findings suggest that TTNs in iodine deficient/sufficient areas predominantly arise from aberrant growth of a single cell. Our results suggest that neither the prevalence of TSHR mutations nor that of monoclonal TTNs is related to iodine supply.

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Introduction

Constitutive activation of the cAMP cascade caused by a somatic thyroid-stimulating hormone receptor (TSHR) mutation results in increased growth and function of the thyroid follicular cells that cause autonomously functioning thyroid tissue (1, 2), which is characterized by its capacity to grow and function independent of the serum levels of thyroid hormones and TSH. Autonomy can present in different histological forms, such as adenoma or adenomatous nodule, but rarely as malignant thyroid epithelial tumors with hyperthyroidism or as microscopic hot areas in euthyroid goiters (2). Thyroid autonomy occurs mostly in toxic multinodular goiters (TMNGs) (2), which is the most frequent cause of thyrotoxicosis in the elderly, especially in iodine-deficient areas (3). In iodine-deficient regions, TMNG and toxic adenoma (TA) account for 50 and 10% of cases with thyrotoxicosis respectively. On the other

hand, in iodine-sufficient areas, thyroid autonomies account only for 3–10% of cases with hyperthyroidism (3). Moreover, the prevalence of thyroid autonomy decreases after the eradication of iodine deficiency (1).

The frequency of somatic mutations in toxic thyroid nodules (TTNs) varies between 8 and 82% for TSHR (1, see TSH Receptor Mutation Database II, <http://www.uni-leipzig.de/~innere> and Table 1 (4–25)), and between 3 and 38% for Gs α (5, 19) in different reports. In particular, studies from Japan with a very high iodine supply have reported distinctly lower frequencies for TSHR mutations in hot nodules (10, 20). Therefore, it has been proposed that apart from differing methodology, differences in iodine intake could account for the variable prevalence of these mutations in different studies (Table 1) (4–25). Although this hypothesis has been questioned recently (20), this study only investigated ten Japanese patients. Therefore, the question whether iodine does influence the prevalence of the

Table 1 Prevalences of the thyroid stimulating hormone receptor (TSHR) and Gs α mutations in studies from different countries in relation to detection methodology and iodine status.

Year of the TSHR mutation analysis (references)	From	Median UI (μ g/l) (references)	Number of TTNs	Method	Type of tissue samples	TSHR region examined	TSHR mutation frequency	Gs α -region examined	Gs α mutation frequency
1990 (4)	USA	145 (23)	4	ASO	FFT or PEB	Not studied		Codon 201 +227	(25%)
1991 (5)	UK	141 (24)	13	ASO	PEB	Not studied		Codon 201 +227	5/13 (38%)
1993 (6)	Belgium	80 (23,24)	11	Sequencing	FFT	ICL3, Residues 589-	3/11 (27%)	Not studied	
1994 (7)	Italy	55–142 (23, 24)	11	Restriction, Sequencing	FNAB	TM5-C Term.	7/11 (64%)	Not studied	
1994 (8)	Germany, Italy		9	Sequencing	FFT	TM5-C Term.	2/9 (22%)	Not studied	
1995 (9)	Italy	55–142 (23, 24)	37	ASO, Sequencing	FFT	ICL 1,2,3 and C Term.	3/37 (8%)	Exons 8 and 9	9/37 (24%)
1995 (10)	Japan	296–728 (25)	45	SSCP	PEB	TM6 and ICL 3	0/45	Not studied	
1996 (11)	Italy	55–142 (23, 24)	44	Sequencing	FFT	Exon 10	9/44 (20%)	Not studied	
1997 (12)	Belgium	80 (23, 24)	33	Sequencing	FFT	Entire coding region	27/33 (82%)	Exons 8 and 9	2/33 (6%)
1997 (13)	Germany	88–148 (23, 24)	31	ASP, Sequencing	FFT	Exons 9 and 10	15/31 (48%)	Exons 7-10	0/31
1997 (14)	Germany	88–148 (23, 24)	13 (inTMNG)	Sequencing	FFT	Exons 9 and 10	5/13 (38%)	Exons 7-10	0/13
1998 (15)	Italy	55–142 (23, 24)	17	Sequencing	FFT	Exon 10	TMNG 5/6 (83%), TA 8/11(72%)	Not studied	
1999 (16)	Italy	55–142 (23, 24)	38	Restriction, Sequencing	FNAB	ICL 3, entire TM	6/38 (16%)	Not studied	
2000 (17)	Germany	88–148 (23, 24)	37 hot areas in 14 patients	Sequencing	PEB	A part of exon 10 (482–677 a.a)	In hot areas 4/37 (18%), in the patients 4/14 (28.5%)	Not Studied	
2000 (18)	Italy	55–142 (23, 24)	20 (in TMNG)	Sequencing	FFT	Exons 9 and 10	14/20 (70%)	Exons 8 and 9	0/20
2001 (19)	Germany	88–148 (23, 24)	75	DGGE, Sequencing	FFT	Exons 9 and 10	43/75 (57%)	Exons 7 and 9	2/75 (3%)
2002 (20)	Japan	296–728 (25)	10	Sequencing	FFT	Entire TSHR coding region	4/10 (40%)	Exons 2–13	1/10 (10%)
2003 (21)	Greece	84–160 (24)	28	Sequencing	FFT	Exons 9 and 10	11/28 (40%)	Exons 7–10	1/28 (4%)
2005 (22)	Turkey	36 (23)	58	SSCP	FFT	A part of exon 10 (482–677 a.a)	10/58 (17%)	Exons 8–9	0/58

SSCP, single strand conformation polymorphism; ASO, allele-specific oligonucleotides; ASP, allele-specific PCR; C-Term, carboxy terminus; TM, Transmembrane; ICL, intracellular loop; DGGE, denaturing gradient gel electrophoresis; TMNG, toxic multinodular goiter; TA, toxic adenoma; AFTN, autonomous functioning thyroid nodule; FNAB, fine needle aspiration biopsy; FFT, fresh-frozen tissue; PEB, paraffin-embedded blocks; TTNs, toxic-thyroid nodules; UI, urinary iodine; aa, amino acid.

TSHR mutations has not been settled, since large numbers of TTNs have so far been investigated only in very few geographic regions with similar iodine status (e.g. Belgium, Italy, Germany, and Greece) (Table 1) (9, 11–13, 16, 19, 21). However, it is not possible to perform the gold standard study which determines the TSHR mutation prevalence in hot nodules with the same methodology for patients of the same population with a clearly documented lifelong exposure to iodine deficiency or normal iodine status. Therefore, to investigate whether the TSHR mutation prevalence is influenced by the degree of iodine deficiency, we determined and compared the prevalence of somatic TSHR mutations in different geographical regions of Turkey with marked differences in iodine supply documented 3–5 years ago. The significant relation between the iodine status and the socioeconomic differences, and the persistence of these differences in the past, makes it highly likely that these differences in iodine supply existed throughout the lifetime of the investigated patients.

Materials and methods

Patients

The present study was approved by the Marmara University Ethics Committee and informed consent was obtained from all patients before surgery.

A total of 74 consecutive TTNs and their surrounding tissues (if not available, peripheral blood samples) were obtained from 56 unrelated patients who underwent subtotal thyroidectomy. One or two hot nodules were found in 38 and 18 of the 56 patients respectively. The mean age of the patients (45 female and 11 male) was 51.5 ± 11.7 ; range, 24–75 years. Histopathology showed nodular hyperplasia (goiter) in all patients. These patients have not been reported previously.

At the time of diagnosis, 53 patients were clinically thyrotoxic. They were treated with propylthiouracil and β -blocker drugs preoperatively. The other three patients who underwent surgical treatment had normal free triiodothyronine (T_3) and free thyroxine (T_4) and suppressed TSH values. Toxic multi- or uninodular goiter was diagnosed on the basis of clinical thyrotoxicosis, thyroid function tests (high T_4 and/or T_3 , and suppressed TSH), thyroid sonography, histopathological examination, and thyroid scintigraphic images associated with predominant Tc^{99m} uptake in one (or more) nodule (nodules) with the suppression of surrounding thyroid tissue.

Nodules defined by scintigraphy and ultrasonography (US) were unequivocally identified intraoperatively. In each case, a map that depicts the nodules as they appeared on the scan in ultrasound and during surgery was drawn. Samples from the center of TTNs, peripheral normal tissues, and blood samples were

taken. Tissue specimens were frozen in liquid nitrogen. The demographic characteristics of the patients are shown in Table 2. In this study, iodine-deficient and sufficient regions were defined as those where the median urinary iodine (UI) secretion was previously determined as $< 100 \mu\text{g/l}$ (26–31). The places of birth of the patients and their areas of residency in Turkey were recorded. We also classified a mixed group because some patients partially lived in both iodine-deficient and -sufficient regions. Istanbul and the region of Kocaeli have been identified as iodine sufficient by representative determinations of the median UI of school children performed by three studies in 1999, 1995, and 1999 respectively (Gür *et al.*, Akpınar *et al.*, and Beykal *et al.*) (27–29). Erdogan *et al.* investigated the UI and goiter prevalence of 5948 school children living in various cities of Turkey. This study was performed from 1997 to 1999 and classified Kastamonu, Bayburt, Trabzon, Erzurum, Malatya, Samsun, Edirne, Aydın, Kayseri, Ankara, Burdur, Isparta, Erzincan, Konya, Van, Diyarbakır, Çorum, Bolu, Kütahya, and Bursa as iodine deficient and endemic goiter regions (26). Further, iodine deficient cities in Turkey were identified by UI determination of school-aged children by other studies (30–31). Since the representative study by Gür *et al.*, identified significant associations of iodine deficiency with socioeconomic variables (27) and because it is known that large socioeconomic differences between Istanbul, Kocaeli and the iodine deficient regions were also present in the past, it is most likely that similar differences in iodine supply which have been documented by the studies described earlier were also present in the past. This assumption is also consistent with an earlier report (32) characterizing Istanbul and Kocaeli as cities with the lowest risk of endemic goiter.

Patients who lived in areas for which UI concentrations cannot be defined on the basis of published results were classified according to the reported occurrence or absence of endemic goiter for these areas. This classification is based on the published prevalence of goiters in the respective areas of residence as detected by palpation (32). The patients classified as iodine deficient on the basis of the reported occurrence of endemic goiters in their areas of residency from 1980 to 1987 are labeled as 'iodine deficient GP' in Table 2. Goiter prevalence is an accepted indicator of iodine deficiency (33). To clearly state the different evidences for iodine deficiency, the iodine-deficient patients were classified into two groups: iodine deficient by UI excretion (iodine deficient UI) and iodine deficient by endemic goiter prevalence (iodine deficient GP). The iodine-deficient patients determined by UI excretion and those classified iodine deficient by the occurrence of endemic goiters in their areas of residency, were compared as two separate groups with the patients living in iodine-sufficient or mixed regions.

Table 2 Demographic and clinical characteristics of the patients.

No.	Age	Gender	TSHR mutation	D727E polymorphism	Clonality
Iodine deficient UI according to references (26, 30, 31) ^a					
1	65	F	D633Y	Positive	Clonal
6	43	F	Negative	Negative	Not informative
15	41	F	V656F	Negative	Clonal
18	45	F	M453T	Positive	Not informative
			T632I	Positive	Not informative
24	72	F	T632I	Negative	Not informative
			L629F	Negative	Clonal
36	43	F	L629F	Negative	Clonal
39	47	F	Negative	Negative	Polyclonal
			Negative	Negative	Polyclonal
40	60	F	P639S	Negative	Clonal
43	35	F	Negative	Negative	Clonal
45	26	F	I568T	Negative	Clonal
49 ^b	42	F	Negative	Positive	Clonal
51	56	F	I630L	Negative	Clonal
			A627V	Negative	Clonal
57	65	F	Negative	Negative	Clonal
61	35	F	Negative	Negative	Not informative
			L512R	Negative	Not informative
62	24	F	F631L	Negative	Clonal
Iodine deficient GP according to reference (32) ^c					
2	37	F	D633Y	Negative	Clonal
7	54	F	Negative	Negative	Not informative
			Negative	Negative	Not informative
9	72	M	Negative	Negative	Male
12	45	F	M453T	Negative	Clonal
16	52	F	L512Q	Negative	Polyclonal
19	50	F	T632A	Positive	Clonal
27	43	M	F631L	Negative	Male
29	64	M	T632I	Negative	Male
31	54	F	T632A	Negative	Clonal
42	48	F	M453T	Negative	Polyclonal
48	59	F	Negative	Negative	Clonal
52	44	F	A428V	Negative	Clonal
			Negative	Negative	Clonal
Iodine sufficient according to references (27–29)					
3	58	F	Negative	Negative	Clonal
5	62	M	T632I	Negative	Male
			A623V	Negative	Male
10	54	F	D619G	Negative	Polyclonal
			D619G	Negative	Clonal
17	31	F	T632I	Positive	Clonal
			L512Q	Positive	Clonal
22	38	F	Negative	Positive	Polyclonal
			I486F	Positive	Polyclonal
25	43	F	D633Y	Positive	Not informative
26	37	F	D619G	Negative	Clonal
28	48	F	T632I	Negative	Not informative
30	57	M	Negative	Negative	Male
33	53	F	S505N	Negative	Clonal
34	75	F	A623V	Negative	Clonal
38	60	F	A623V	Negative	Not informative
41	72	F	Negative	Negative	Clonal
			Negative	Negative	Clonal
47	42	M	D633H	Negative	Male
Mixed according to references (26–31)					
8	58	M	Negative	Negative	Male
			Negative	Negative	Male
11	71	F	D619G	Positive	Clonal
20	54	F	Negative	Negative	Clonal
			M453T	Negative	Polyclonal
21	48	F	M453T	Negative	Clonal
			L629F	Negative	Clonal
23	66	M	I486N	Negative	Male

Table 2 Continued

No.	Age	Gender	TSHR mutation	D727E polymorphism	Clonality
32	62	F	I486F A623V A623V	Negative Positive Positive	Male Polyclonal Polyclonal
35	41	F	T632I	Negative	Clonal
44	50	M	I640K A428V	Negative Negative	Male Male
46	47	F	Negative	Positive	Polyclonal
50	43	F	M453T	Negative	Polyclonal
53	53	M	S281N	Negative	Male
54	55	F	D619G	Positive	Polyclonal
55	51	F	T632I	Negative	Clonal
56	60	M	Negative	Positive	Male
60	52	F	S281N	Negative	Polyclonal

A total of 74 consecutive toxic thyroid nodules (TTNs) were obtained from 56 unrelated patients who underwent thyroidectomy.

^aIodine deficient UI, iodine deficient by urinary iodine excretion. ^bGs α mutation (R201H) has been detected in only one patient. ^cIodine deficient GP, iodine deficient by endemic goiter prevalence.

DNA isolation Genomic DNA was isolated from frozen-tissue specimens using standard techniques as described previously by Krohn *et al.* (17). Genomic DNA from lymphocytes was isolated using a standard phenol-chloroform methodology.

PCR, denaturing gradient gel electrophoresis (DGGE) and sequencing of TSHR gene In order to investigate the TSHR sequence for mutations in exons 9 and 10 of the TSHR gene, PCR, DGGE, and sequencing analysis were performed as described by Trülsch *et al.* (19). The PCR products in which mutations were detected in exons 9 and 10 of the TSHR gene by DGGE were sequenced using Big Dye-terminator chemistry (Applied Biosystems, Germany) according to the manufacturer's instructions and analyzed on an automatic sequencer ABI 310 (Applied Biosystems, Germany).

PCR and sequencing of Gs α DNA samples without TSHR gene mutations were analyzed for known Gs α mutations in exons 7 and 8. PCR and sequencing reactions were performed as described earlier using the following primers: sense 5' AGTTGGCAAATTGATGTGAGC 3' and antisense 5' TCTCTATAAACAGTGCA-GACC 3'.

Clonality and quantification of X-chromosome inactivation Clonality was determined and analyzed as described by Krohn *et al.* (34).

Total RNA extraction Total RNA of the tissue sample carrying a new mutation was isolated from frozen-tissue specimens using TRIzol reagent (Life technologies, USA) according to the manufacturer's manual. Later, the total RNA was purified with the

RNeasy kit (Qiagen, Germany) according to the manufacturer's protocol.

Cloning of new TSHR mutations Mutations I486N, A428V, A627V, and I640K were introduced into the human TSHR via site-directed mutagenesis. Human TSHR-pSVL was used as a template. PCR products containing the mutations were digested with the restriction enzymes (MBI Fermentas) BspTI and Eco91I (I486N and A428V) or Eco81I and Eco91I (A627V and I640K). The obtained fragments were used to replace the corresponding part in the wild-type (wt) TSHR-pSVL vector. Mutated TSHR sequences were verified by dideoxy sequencing as described earlier.

Cell culture and transient expression of mutant TSHR COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin (Gibco Life technologies) at 37 °C in a humidified 5% CO₂ incubator. Cells were transiently transfected using the GeneJammer Transfection reagent (Stratagene, NL). For determination of cAMP, accumulation cells were transfected in 24-well plates (0.5 \times 10⁵ cells per well) with 0.5 μ g DNA per well. For FACS analysis, cells were transfected in 12-well plates (1 \times 10⁵ cells per well) with 1 μ g DNA per well.

cAMP accumulation assay Activation of cAMP cascade was performed 48 h after transfection. Cells were preincubated with serum-free DMEM without antibiotics containing 1 mM 3-isobutyl-1-methylxanthine (IBMX) (Sigma Chemical Co., USA) for 30 min at 37 °C in a humidified 5% CO₂ incubator. Subsequently, cells were stimulated in the same medium supplemented with 100 mU/ml bTSH (Sigma Chemical Co.) for 1 h.

Reactions were terminated by aspiration of the medium. The cells were washed once with ice-cold PBS and lysed by the addition of 0.1 N HCl (equivalent to 0.1 M). The supernatants were collected and dried. cAMP content of the cell extracts was determined using the cAMP AlphaScreen assay (PerkinElmer Life Sciences, Belgium) according to the manufacturer's instructions. Basal cAMP accumulation of wtTSHR was set at 1 and results of the mutants (basal and stimulated cAMP content) were calculated according to this.

Effect of TSHR expression on basal cAMP accumulation (linear regression analysis of constitutive activity as a function of TSHR expression) COS-7 cells were transiently transfected in 24-well plates (0.5×10^5 cells per well) with six concentrations of weightand TSHR constructs (50, 100, 150, 200, 250, and 300 ng DNA per well) for radioligand binding and in parallel for the cAMP accumulation assay. For radioligand binding assays, cells were incubated for 4 h at room temperature in the presence of 180,000–200,000 c.p.m. of ^{125}I -bTSH supplemented with 5 mU/ml non-labeled bTSH. The effect of expression level on basal cAMP accumulation was analyzed using the results for TSH binding and basal cAMP accumulation obtained for the six DNA concentrations to calculate the specific constitutive activity of the mutants. Slopes were determined by plotting TSH binding (*x*-axis) versus basal cAMP accumulation (*y*-axis) using the linear regression function of Graph Pad Prism 2.01 for Windows. The slope of the wtTSHR was set at 1 and slopes of the mutants were calculated according to this. The data for each of the constructs represent results from at least two independent experiments, each performed in duplicate.

FACS analysis Cells were detached from the dishes 48 h after transfection using 1 mM EDTA and 1 mM EGTA in PBS and transferred in Falcon 2054 tubes. Cells were washed once with PBS containing 0.1% BSA and 0.1% NaN_3 before incubation with a 1:200 dilution of a mouse antihuman TSHR monoclonal antibody 2C11 (MAK 1281, Linaris, Wertheim-Bettingen; 10 $\mu\text{g}/\text{ml}$) for 1 h. The cells were washed twice and incubated at 4 °C for 1 h in darkness with 1:200 dilution of fluorescein-conjugated F(ab)2 rabbit anti-mouse IgG (Serotec, Oxford, UK). Cells were washed twice and fixed with 1% paraformaldehyde before FACS analysis (FACScan Becton Dickinson and Co., Franklin Lakes, NJ). Receptor expression was determined by fluorescence intensity, whereas the percentage of signal-positive cells corresponds to transfection efficiency.

Statistics Statistical analysis was carried out by one-way ANOVA, followed by Dunnett's Multiple Comparison Test using Graph Pad Prism 2.01 for Windows (not significant, $P > 0.05$; significant, $P = 0.01$ – 0.05 ; very significant, $P = 0.001$ – 0.01). χ^2 -test was used to compare the frequency of TSHR and $\text{Gs}\alpha$ mutations and the TSHR polymorphism in different regions.

Results

Identification of mutations, polymorphisms, and clonality All detected TSHR and $\text{Gs}\alpha$ gene mutations are summarized in Table 3. Over all, in 74 TTNs from 56 patients, 52 (70.2%) TSHR mutations were found. All the mutations were heterozygous and detectable in adenoma tissues but not in the surrounding tissues. Most of the mutations are located in the transmembrane helix (TMH) 6 (Table 3). Of all detected mutations, 49 are known as constitutively active. Recently, one of them located at position A428V (GCT→GTT) in TMH 1 (Table 3) has been described as a germline mutation (35). However, A428V has not been characterized so far. Three new TSHR mutations (A627V, I640K, and I486N) which have not been reported or characterized previously

Table 3 TSHR and $\text{Gs}\alpha$ gene mutations in the 74 toxic thyroid nodules (TTNs).

Amino acid position and substitution	Base substitution	Mutation frequency	Mutation position
S281N	AGC → AAC	1	ECD
S281T	AGC → ACC	1	ECD
A428V ^a	GCT → GTT	2	1st TMH
M453T	ATG → ACG	6	2nd TMH
I486F	ATC → TTC	2	1st ECL
I486N ^b	ATC → AAC	1	1st ECL
S505N	AGC → AAC	1	3rd TMH
L512Q	CTG → CAG	2	3rd TMH
L512R	CTG → CGG	1	3rd TMH
I568T	ATC → ACC	1	2nd ECL
D619G	GAT → GGT	5	3rd ICL
A623V	GCC → GTC	5	3rd ICL
A627V ^b	GCT → GTT	1	6th TMH
L629F	TTG → TTC	2	6th TMH
L629F	TTG → TTT	1	6th TMH
I630L	ATC → CTC	1	6th TMH
F631L	TTC → TTA	2	6th TMH
T632I	ACC → ATC	8	6th TMH
T632A	ACC → GCC	2	6th TMH
D633Y	GAC → TAC	3	6th TMH
D633H	GAC → CAC	1	6th TMH
P639S	CCA → TCA	1	6th TMH
I640K ^b	ATC → AAA	1	6th TMH
V656F	GTT → TTT	1	3rd ECL
R201H ($\text{Gs}\alpha$)	CGT → CAT	1	

Over all, 52 (70.2%) TSHR mutations were found in 74 TTNs from 56 patients.

^aOne of these has been reported recently, but not characterized so far.

^bThree of these are novel mutations not reported or characterized so far. ECD, extracellular domain; ECL, extracellular loop; ICL, intracellular loop; TMH, transmembrane helix.

were also detected in this study. One of the new constitutively activating TSHR mutations is located at position I486N in the extracellular loop (ECL) 1. The other two new mutations are located near the hot spot region in TMH 6 at position A627 (GCT \rightarrow GTT; A627V) and I640 (ATC \rightarrow AAA; I640K).

DNA samples without detectable TSHR gene mutations were also screened for constitutively activating Gs α mutations at exons 7 or 8. One heterozygous Gs α mutation located at position R201, leading to an exchange of arginine to histidine, was identified in a hot nodule.

In 12 patients (21.4%), the polymorphism D727E at the intracellular cytoplasmic tail of the TSHR was found in the nodular tissue and also in the DNA extracted from the lymphocytes. In 11 patients, the polymorphism was heterozygous and 1 was found homozygous. This latter patient also carried the D633Y mutation, located in the sixth transmembrane domain of the TSHR.

Clonal origin was studied in 59 TTNs from 45 female patients using X-chromosome inactivation. In this group, 38 patients with 48 nodules were heterozygous for the (CAG)_n-polymorphism in exon 1 of the human androgen receptor (HUMARA). Seven patients with eleven nodules were not informative for this polymorphism. Non-random X-chromosome inactivation was detected in 34 (70.9%) of 48 cases, whereas the other 14 (29.1%) showed random inactivation indicating a polyclonal origin. In the group of TTNs that contains a somatic mutation in the TSHR or Gs α , 26 of 36 (72.2%) cases showed non-random X-chromosome inactivation characterized by a loss of amplification for 1 allele after PCR from HpaII-digested DNA. In 10 of 36 (27.8%) cases with a TSHR mutation, random X inactivation was detected. In the group of TTNs without detectable mutations in the TSHR or Gs α , only 4 of 12 (33.3%) cases showed random inactivation, 8 of 12 (66.7%) toxic nodules without TSHR mutations showed non-random X-chromosome inactivation.

Prevalences of the TTNs with somatic mutations, clonality and D727E polymorphism in the iodine-deficient or -sufficient regions In 74 TTNs, 52 (70.2%) TSHR mutations were found. Contamination or sampling imprecisions are unlikely reasons for the detection of the same mutation in the 2 nodules of patients 10 as suggested by reanalysis of the samples. For patient 32, a sampling problem could not be definitely excluded. The prevalence of TSHR mutations was similar in the iodine-deficient UI, iodine sufficient, mixed and iodine deficient GP groups. Although the prevalence of TSHR mutations in the iodine-sufficient region (73.7%) was slightly higher than in the iodine-deficient UI (70%) and GP (64.3%), the difference was not statistically significant (χ^2 , 0.652; *P*, 0.884) (Table 4).

The polymorphism D727E at the intracellular cytoplasmic tail was present in 12 (21.4%) patients.

Table 4 Prevalence of somatic TSHR and Gs α mutations in toxic thyroid nodules in iodine-sufficient and -deficient areas.

Mutation	Iodine deficient UI	Iodine deficient GP	Iodine sufficient	Mixed
TSHR/Gs α Positive	14 (70%)	9 (64.3%)	14 (73.7%)	16 (76.2%)
TSHR/Gs α Negative	6 (30%)	5 (35.7%)	5 (26.3%)	5 (23.8%)

The prevalence of TSHR mutations was similar in the iodine-deficient UI, iodine sufficient, iodine deficient GP, and mixed groups (χ^2 : 0.652, *P*: 0.884). The results of the statistical tests for specific group differences were found non-significant (between iodine-deficient UI and iodine-sufficient groups χ^2 , 0.065; *P*, 0.798, between iodine deficient GP and iodine-sufficient groups χ^2 , 0.337; *P*, 0.561). When iodine-deficient UI and iodine-deficient GP considered as one group, no statistical difference was found between iodine sufficient and (iodine-deficient UI + iodine-deficient GP) groups (χ^2 : 0.51 and *P*: 0.771).

The prevalence of polymorphism D727E was found to be 20% (3/15) in iodine-deficient UI, 21.4% (3/14) in iodine sufficient, 8.4% (1/12) in iodine deficient GP, and 33.4% (5/15) in the mixed groups. The prevalence of this polymorphism did not differ between groups (χ^2 , 2.503; *P*, 0.475).

Forty-eight cases were heterozygous for the (CAG)_n in exon 1 of the HUMARA gene and 11 were not informative for the polymorphism. Non-random X-chromosome inactivation was shown in 34 (70.9%) of 48 cases, whereas the other 14 (29.1%) showed random inactivation indicating polyclonal origin. The prevalences of clonal nodules were found to be 85.7% (12/14) in the iodine-deficient UI group, 77.7% (7/9) in iodine-deficient GP group, 75% (9/12) in the iodine-sufficient group, and 46% (6/13) in the mixed group. There was no statistically significant difference between the prevalences of nodules with clonality in the groups (χ^2 , 5.644; *P*, 0.130) (Table 5).

Functional analysis of new mutations After transient transfection in COS-7 cells, functional characterization of the TSHR mutants was performed by the determination of cell surface expression, basal and bTSH-induced cAMP accumulation, as well as specific constitutive activity. Functional characteristics of all new somatic mutants are summarized in Table 6.

A627V, I640K, and I486N were characterized by a cell surface expression in a range of 44–61%, compared with the wt TSHR (set at 100%), whereas A428V revealed the lowest expression with 25%. However, all mutants caused constitutive activation (three- to sevenfold over wt TSHR basal). Slopes were determined to evaluate all mutants independently from their cell surface expression. The highest slope with 88.2 revealed mutant A428V, followed by I640K with 14.6, I486N with 6.7, and A627V with 4.2 (Table 6). Activation of the mutated TSHRs showed a reduced cAMP response only for A627V and I640K.

Table 5 Frequency of clonal or polyclonal origin of the toxic thyroid nodules in iodine-sufficient and -deficient areas.

	Regions				TSHR/Gs α mutation	
	Iodine-deficient UI	Iodine-deficient GP	Iodine-sufficient	Mixed	TSHR/Gs α negative	TSHR/Gs α positive
Number of informative females studied	14	9	12	13	12	36
Clonal origin	12 (85.7%)	7 (77.7%)	9 (75%)	6 (46%)	8 (66.7%)	26 (72.2%)
Polyclonal origin	2 (14.3%)	2 (22.3%)	3 (25%)	7 (54%)	4 (33.3%)	10 (27.8%)

TSHR, thyroid stimulating hormone receptor; UI, urinary iodine; GP, goiter prevalence. There was no significant difference between the prevalences of nodules with clonality in the iodine-deficient UI, iodine sufficient, iodine-deficient GP and mixed groups (χ^2 : 5.644 P : 0.130).

Discussion

Prevalences of somatic mutations of TTNs in relation to the iodine status Activating somatic mutations of the TSHR and Gs α have been described with different frequencies (Table 1). Apart from the sensitivity of the detection methods, the size of the TSHR region screened for mutations, the number of nodules examined, the quality of the tissue samples, and the differences in iodine supply are possible explanations for this variability of the prevalence of somatic TSHR mutations (1).

Therefore, this study determined the TSHR mutation frequency in TTNs using a very sensitive method (DGGE) covering exons 9 and 10 of the TSHR in regions of Turkey with sufficient or insufficient iodine supply and compared these results with the TSHR mutation frequencies from other countries with various degrees of iodine supply. This study also tried to answer the question of whether different degrees of iodine supply in Turkey, which were very likely present throughout the lifetime of the investigated patients, do influence the prevalence of TSHR mutations in hot nodules.

Table 6 Functional characterization of the previously reported (35) A428V and new somatic thyroid stimulating hormone receptor (TSHR) mutations. Data are given as mean \pm s.e.m. of three independent experiments, each carried out in duplicate.

Transfected construct	Cell surface expression FACS % of wt TSHR	cAMP accumulation		
		Relative to wt basal		Fold over wt basal Slopes
		Stimulated	Basal	
wt TSHR	100	1	15.1 \pm 0.6	1
A428V	25.1 \pm 0.6 [†]	6.4 \pm 1.0 [†]	13.6 \pm 2.4	88.2 \pm 2.1 [†]
I486N	60.9 \pm 1.3 [†]	7.1 \pm 1.2 [†]	12.9 \pm 4.3	6.7 \pm 1.4 [†]
A627V	52.0 \pm 1.1 [†]	3.2 \pm 0.7*	8.2 \pm 0.8*	4.2 \pm 1.0 [†]
I640K	44.2 \pm 1.6 [†]	5.9 \pm 0.4 [†]	8.0 \pm 0.4*	14.6 \pm 2.2 [†]

For determination of cell surface expression, cAMP accumulation and linear regression analysis of constitutive activity as a function of TSHR expression (see Materials and methods) mutated TSHRs were cloned into the expression vector pSVL and transiently expressed in COS-7 cells. The wild-type (wt) receptor and empty pSVL vector were used as controls. Statistical analyses were carried out by one-way ANOVA, followed by Dunnett's Multiple Comparison Test using Graph Pad Prism 2.01 for Windows (not significant $P > 0.05$; significant (*) $P 0.01-0.05$; very significant ([†]) $P 0.001-0.01$).

From the 56 patients, 74 TTNs were examined and 52 (70.2%) TSHR mutations were found. The mutations found in our study are distributed along the exons 9 and 10 of the TSHR (Table 3). All new TSHR mutations (A627V, I640K, and I486N) showed constitutive activity when transiently expressed in COS-7 cells (Table 6). Only 1 of the 74 nodules harbored a Gs α mutation (R201H).

Different frequencies of TSHR mutations were reported in different countries ranging from 8 to 77% (7, 9, 11, 15, 16, 18) in Italy (median urine iodine level, 55–122 mcg/l), 82% in Belgium (12) (median UI level, 80 mcg/l), 48–57% in Germany (13, 19) (median UI level, 88–148 mcg/l), and 40% in Greece (21) (median UI level, 84–160 mcg/l). On the other hand, only somatic TSHR mutations were found in 1 of 45 TTNs in Japan (median UI level, 296–728 mcg/l), a country with high iodine supply (10). The frequencies of TSHR mutations were described to be high in Italy (64, 77, 70%) (7, 15, 18) and Belgium (82%) (12) and slightly lower in Germany (48, 57%) (13, 19) and Greece (40%) (21). However, apart from this possible relation of the TSHR mutation frequency to the slight differences in iodine supply, the frequency of the TSHR mutation was also high in those studies in which exons 9 and 10 were screened by direct sequencing or DGGE in fresh-frozen tissues (12–15, 18–19).

In this study, there were no significant differences regarding the TSHR mutation frequencies between iodine-sufficient and -deficient regions (Table 4). The prevalence of somatic TSHR mutations in TTNs was 70% in iodine-deficient UI, 73.7% in iodine sufficient, 64.3% in iodine-deficient GP, and 76.2% in mixed regions respectively. There was no statistically significant difference between the prevalence of mutations in iodine-deficient UI (70%), iodine deficient GP (64.3%), iodine sufficient (73.7%), and mixed (76.2%) groups. This suggests that the etiology of TTNs in both iodine-deficient and -sufficient areas predominantly involves mutagenesis of the TSHR. Constitutively activating TSHR mutations seem to be the most frequent molecular event independent of the status of iodine supply. This does not contradict evidence that deficient iodine supply increases the frequency of nodular transformation (36), since our sample of patients with TTNs does not allow us to infer such a frequency.

It rather suggests that constitutive activation of the cAMP cascade is a prerequisite for TTNs and is most frequently achieved through TSHR mutagenesis irrespective of the iodine status.

Our patients were classified as iodine deficient, sufficient, or mixed group based on published UI excretion and endemic goiter prevalence (26–32). We are aware that this approach might be prone to a certain percentage of misclassification of patients (37–39). However, the alternative approach to using individual spot UI values is likely to be highly variable over time (40). Seasonal, diurnal, and day-to-day variations of UI excretions have been reported (41). Therefore, individual UI determinations are unreliable as an indicator for an individual's iodine status. However, most important, it would be impossible to observe iodine-deficient patients for many years without suggesting them to use iodized salt. Therefore, the use of published UI excretion or goiter population screening data in areas with stable social and economic circumstances and low mobility (42) appears as the most reliable and feasible basis for an examination of the possible influence of iodine status on the TSHR mutation frequency in hot nodules.

The TSHR mutation frequency of all subgroups in this study is higher than that in the recent study from Japan with 40% (20) and higher compared with other studies in Germany (48, 57%) (13, 19) or Greece (40%) (21) and Italy (64, 70%) (7, 18), examining at least exons 9 and 10. Some of the variability of the TSHR mutation frequency might be attributable to methodological differences, for example, sequencing (8, 11, 14, 20–21) versus DGGE (19) as previously shown by Trültzsch *et al.* who could detect 6 of the 43 somatic TSHR mutations only with DGGE, but not direct sequencing of the tissue DNA. This might have obscured some of the possible iodine status-related differences. However, also the comparison of this study with the other largest hot nodule group, which both examined with the same sensitive method (DGGE) shows differences for TSHR mutation frequencies (70.2 vs 57%, but not significant χ^2 , 3.31; $P > 0.05$, 19) which are of a similar magnitude to the above differences for patients with a similar iodine status (70.2 vs 40% for Greece, χ^2 , 9.08; $P < 0.01$, 21), (70.2 vs 70% for Italy χ^2 , 0.020, $P > 0.05$, 18) and (70.2 vs 82% for Belgium, χ^2 , 1.15, $P > 0.05$, 12). Given the methodological differences, imprecisions regarding the lifelong iodine status of the patients and the limited numbers of hot nodules samples examined, it is therefore very likely that we are presently unable to detect any significant differences in somatic TSHR mutation frequencies related to the iodine status. However, it could also be possible that the relatively small differences in UI within Europe are a reason for this somatic TSHR mutation frequency variability among European countries, since the association of large

differences in hyperthyroidism (due to thyroid autonomy) with small differences in iodine intake has been reported (43).

We found that most of the TTNs (26 of 36, 72.2%) containing a TSHR or Gs α mutation were of monoclonal origin. In line with previous studies reporting non-random X-chromosome inactivation in 79 and 70% respectively (19, 34), these results demonstrate that TTNs with a somatic mutation in the TSHR are predominantly monoclonal. In the present study, ten (27.8%) mutation-positive nodules showed polyclonal origin. For the mutation-positive nodules, a frequency of 100% of clonal origin should be reached. However, due to the limited sensitivity of the assay, contamination by polyclonal cells from blood or by connective tissue and unaffected cells will lower the chance to get this 100% (34). Moreover, the error rate of the assay is around 5–10% (34). Most of the TTNs (8/12, 66.7%) without a mutation were also monoclonal. Therefore, mutations in other candidate genes of the cAMP signal transduction pathway might play a role in these monoclonal TTNs without TSHR mutations. The most relevant finding is the similar frequency of a clonal origin for both iodine-deficient UI (85.7%), GP (77.7%) and iodine-sufficient regions (75%). This also supports the conclusion drawn from the somatic mutation frequency, which is 73.7% for the iodine-sufficient regions and 70 and 64% for the iodine-deficient UI and GP regions respectively. Both these findings suggest that TTNs in both iodine-deficient and -sufficient areas predominantly arise from aberrant growth of a single cell. In the hot nodules of some patients (e.g. 10 and 32), we detected somatic TSHR mutations which indicate a neoplastic growth in combination with results of the clonality assay that suggest a polyclonal origin. This contradiction very likely stems from differences in the sensitivity of the methods used for clonal analysis and detection of mutations. The PCR-based clonality assay would indicate a polyclonal origin even if the tumor tissue is monoclonal when contamination with non-tumor tissue is more than about 30% (34). With DGGE, a mutation in the TSHR is still detectable when contamination is more than 90% (44). The cases where a somatic TSHR mutation was detected but X-chromosome analysis indicates polyclonality are very likely cases with a higher degree of contamination with blood, connective, and surrounding healthy tissue.

Taken together, this study demonstrates that the frequency of somatic TSHR mutations in hot nodules is actually high in Turkey without a significant difference between iodine-deficient and -sufficient regions. Moreover, the frequency of a monoclonal origin of TTNs is also high. Our results suggest that within the limitation discussed above, the proportion of neither TSHR mutations nor that monoclonal origin of TTNs is related to the iodine supply in the diet.

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