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Mutations in the Thyrotropin Receptor Signal Transduction Pathway in the Hyperfunctioning Thyroid Nodules from Multinodular Goiters: A Study in the Turkish Population

HULYA GOZU, MELIKE AVSAR*, RIFAT BIRCAN*, SERAP SAHIN*, OGUZHAN DEYNELI, BEYAZIT CIRAKOGLU* AND SEMA AKALIN

Section of Endocrinology and Metabolism, Department of Medicine, Marmara University Medical School, Istanbul 81190, Turkey
**Department of Medical Biology, Marmara University Medical School, Istanbul 34668, Turkey*

Abstract. Many studies have been carried out to determine $G_s\alpha$ and TSHR mutations in autonomously functioning thyroid nodules. Variable prevalences for somatic constitutively activating TSHR mutations in hot nodules have been reported. Moreover, the increased prevalence of toxic multinodular goiters in iodine-deficient regions is well known. In Turkey, a country with high incidence rates of goiter due to iodine deficiency, the frequency of mutations in the thyrotropin receptor signal transduction pathway has not been evaluated up to now. In the present study, a part of the genes of the TSHR, $G_s\alpha$ and the catalytic subunit of the PKA were checked for activating mutations. Thirty-five patients who underwent thyroidectomy for multinodular goiters were examined. Genomic DNAs were extracted from 58 hyperactive nodular specimens and surrounding normal thyroid tissues. Mutation screening was done by single-strand conformational polymorphism (SSCP) analysis. In those cases where a mutation was detected, the localization of the mutation was determined by automatic DNA sequencing. No $G_s\alpha$ or PKA mutations were detected, whereas ten mutations (17%) were identified in the TSHR gene. All mutations were somatic and heterozygotic. In conclusion, the frequency of mutations in the cAMP signal transduction pathway was found to be lower than expected in the Turkish population most likely because of the use of SSCP as a screening method and sequencing only a part of TSHR exon 10.

Key words: Thyroid nodules, Thyrotropin receptor, $G_S\alpha$ gene, Protein kinase A gene, Mutations

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SPECIFIC alterations of growth factors, growth factor receptors, oncogenes and tumour suppressor genes have been identified in thyroid neoplasia [1]. Toxic thyroid adenoma is a solitary benign thyroid neoplasm that produces thyroid hormones independent from TSH [2]. Multinodular goiter (MNG) is a common thyroid disease in endemic areas. Structural and functional heterogeneity is the most characteristic hallmark of MNG [3]. It is encountered in a wide spectrum of specimens ranging from a single hyperfunctioning nodule within an enlarged thyroid gland which has additional nonfunctioning nodules, to multiple hyperfunctioning nodules [4]. Many factors are involved in the etiology of MNG which may become autonomous and finally toxic [2].

The thyrotropin receptor (TSHR) is a member of the large family of G protein-coupled receptors [5–7]. After activation, the TSHR binds to the heterotrimeric G protein complex [7, 8], inducing the exchange of GDP with GTP. Thus the G protein is separated into its α and $\beta\gamma$ subunits. $G_s\alpha$ stimulates the adenylate cyclase pathway, which leads to the formation of cAMP [5, 7, 8] and subsequently to the activation of protein kinase A (PKA). PKA consists of two regulatory (R) and two catalytic (C) subunits. When two cAMP molecules bind to the R subunit, active C subunits are released

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Correspondence to: Hulya GOZU, M.D., Kozyatagi Mah. Beyaz Kanarya Sok. Inmak Sitesi. Sena Apt. B Blok. Daire: 5, Erenköy, Istanbul 43742, Turkey

and enter the nucleus where they phosphorylate CREB, which is involved in the transcription regulation process [7, 9].

Therefore, both thyroid growth and function are regulated by cAMP-PI_3 [5]. Constitutive activation of the TSHR signal transduction pathway has been reported in toxic thyroid adenomas and MNG. This activation may be caused by somatic mutations in the $G_s\alpha$ subunit or TSHR gene [1]. Somatic mutations affecting the cAMP signal transduction pathway were first detected in $G_S\alpha$ gene [10]. $G_S\alpha$ mutations have been found to be responsible for a small portion of autonomously functioning thyroid nodules with varying frequencies ranging from 3 to 38% [11, 12]. In two separate studies in which allele specific oligonucleotide probing (ASO) was used, $G_S\alpha$ mutations were reported in 5 (38%) of 13 and 9 (24%) of 37 autonomous functioning thyroid nodules [12, 13]. In other studies, which mostly used direct sequencing or SSCP, $G_S\alpha$ mutations were not found in thyroid nodules [14–18].

Several studies have documented the presence of activating mutations TSHR gene with varying frequencies ranging from 8 to 86% [13, 14] in benign autonomous functioning thyroid adenomas. In subsequent studies, TSHR mutations were also detected in hyperfunctioning nodules from patients with toxic MNG with frequencies up to 83% [15, 16, 19–22]. Mutations of the TSHR and G proteins provide satisfactory explanations for a variety of thyroid diseases. However, most cases of nodular thyroid disease do not exhibit activating mutations in TSHR or $G_s\alpha$ genes. Thus research into the signal transduction pathways downstream of the G proteins became important [8]. Based on this theory, a part of the gene of the catalytic subunit of the PKA was also screened for mutations in thyroid nodules [9]. Catalytic (C) subgroup mutants of PKA were first identified in 1992 [23]. These mutations have no effect on catalytic activities but block the inhibitory effect of the regulatory subunit [24]. It was thought that they were involved in the pathogenesis of thyroid nodules. Therefore, a part of the gene of the catalytic subunit of PKA was also screened for mutations in thyroid nodules, but none was detected [9].

It is reported that the incidence of mutations is higher in thyroid nodules from iodine deficient areas than from iodine sufficient areas [14, 16, 17, 22, 25]. Iodine deficiency is an important public health problem in Turkey. Legislation for the mandatory iodization of household salt was passed in 1999 and strictly enforced in 2000 [26]. Goiter prevalence was reported as high as 30.5% in 1988 [27] and as 31.8% in 2002 [26]. To date, three TSHR mutations have been identified in Turkish population as case reports. A Leu 512 Arg mutation has been investigated in an autonomously functioning papillary carcinoma [28]. Two new mutations in two hyperfunctioning thyroid nodules have also been identified recently in Turkey [29]. But hyperfunctioning thyroid nodules have not been screened for constitutively activating mutation before. Therefore, this study was designed to evaluate the frequency of mutations in a part of the genes of the TSHR, $G_s\alpha$ and the catalytic subunit of the PKA in hyperfunctioning thyroid nodules from patients with MNG in Turkey, a country with iodine deficiency.

Materials and Methods

Patients

The present study was approved by the Marmara University Ethics Committe. Informed consent was obtained from patients before surgery. Thirty-five patients (mean age, 45.4 ± 14.51; range, 18–70 yr) who underwent thyroidectomy for toxic or nontoxic multinodular goiters were examined. Thyroid function tests (Total T_4, T_3, TSH), anti thyroid peroxidase antibodies (Anti-TPO), thyroid ultrasonographies (available in 23 patients) and thyroid scintigraphic images using Tc^{99m} were evaluated. Their place of birth and habitation in Turkey were recorded. The endemic goiter regions in Turkey were described based on the results of previous studies [26, 27, 30].

Thyroid function tests were evaluated. Accordingly the patients were classified as euthyroid, hyperthyroid or subclinical hyperthyroid with normal fT3 and fT4 levels and serum TSH values lower than 0.1 mU/L.

Images were evaluated 15 min after a tracer dose of 3.5 mCi Tc-99m pertechnate. According to thyroid scintigraphic images, nodules were classified as hyperactive and hypoactive, respectively.

Diagnosis of toxic MNG was based on the findings of thyroid function tests (high T_4 and/or T_3 and suppressed TSH). Hyperfunctioning/nonfunctioning areas were identified by thyroid scintigraphy and histopathological examination.

Diagnosis of nontoxic MNG was based on the findings of thyroid function tests (euthyroid or subclinical

hyperthyroid). Hyperfunctioning/nonfunctioning areas were identified by thyroid scintigraphy and histopathological examination.

Twenty-one patients were clinically thyrotoxic at the time of diagnosis. These patients were treated with propylthiouracil and β blocker drugs preoperatively. Lobectomy, subtotal or near total thyroidectomy were performed in all patients. Histopathology showed nodular hyperplasia (goiter) in all patients.

The demographic characteristics of the patients and their clinical parameters are shown in Table 1.

Methods

DNA isolation

The nodular and surrounding tissues were analyzed by pathology. Nodules were identified by matching palpation, thyroid ultrasonographies (if available), scintigraphies and macroscopic findings of surgical specimens. Specimens of 58 hyperactive thyroid nodules and 35 adjacent normal thyroid tissue of patients were obtained after surgery and stored at –70°C. Peripheral blood was collected in EDTA and stored at –20°C.

Extraction of genomic DNAs from the frozen tissue specimens and lymphocytes was performed using the phenol-chloroform method as described previously [31, 32].

Polymerase chain reaction (PCR)

The regions known to harbor activating mutations; exons 8–9 in $G_s\alpha$, a part of exon 10 in the TSH receptor were amplified using primers and PCR conditions as described previously [18, 33]. A part of area including codon 196 of the PKA gene was amplified using primers and PCR conditions as described by Esapa and Harris [9].

Single-strand conformational polymorphism (SSCP)

In order to detect mutations in a part of exon 10 of the TSHR gene, exons 8–9 of $G_s\alpha$ and a region including codon 196 of the catalytic subunit of the PKA gene, SSCP analysis was performed using previously reported methods with slight modifications [34]. The PCR product was diluted in DNA loading buffer by adding three µl of the PCR reaction to 15 µl of 95% formamide and 10 mM NaOH. These mixtures were denaturated at 95°C for 10 min. After that the samples were immediately chilled on ice and loaded onto a 12% polyacrylamide gel. Gels were run at 180 V and 25 mA overnight at room temperature and then were stained with 0.1% silver nitrate. All samples were assayed using at least two different conditions.

Sequencing

Sequencing reactions were analysed with an automatic sequencer. We sequenced PCR products, in which mutations were detected by SSCP analysis by using Sequenase II and the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences, USA) on a ABI Prism 310 Genetic Analyzer (Applied Biosystems, USA) according to the manufacturer's manual.

Statistical analysis

Statistical analysis was done using Fischer test.

Table 1. Demographic characteristics and clinical parameters of all patients

Age (years)		18–70 (45.4 ± 14.51)
Gender	Female	30 (85.8%)
	Male	5 (14.2%)
Mean duration of goiter (years)		10.86 ± 11.77
Living in endemic goiter region	Ever	28 (80%)
	Never	7 (20%)
Family history of nodular thyroid diseases	Positive	21 (60%)
	Negative	14 (40%)
Thyroid disorder	Toxic multinodular goiter	21 (60%)
	Nontoxic multinodular goiter	14 (40%)
Thyroid function	Hyperthyroid	21 (60%)
	Subclinical hyperthyroid	3 (8.5%)
	Euthyroid	11 (31.4%)

Results

Identification of mutations

Genomic DNA was isolated from 93 thyroid tissue specimens (58 hyperactive thyroid nodules and 35 normal tissue samples) and peripheral leucocytes. A part of exon 10 including about 70% of all currently known mutations of the TSHR gene [35] (TSH Receptor mutation Database II, http://www.uni-leipzig.de/~innere/), exon 8 and 9 of the $G_S\alpha$ gene and codon 182–213 of the catalytic subunit of the PKA gene were amplified by PCR. Then SSCP was applied to all samples. No alterations were detected in the exon 8–9 of $G_S\alpha$ and codon 182–213 of the catalytic subunit of the PKA gene.

On the other hand, 10 samples from 58 hyperfunctioning nodules revealed DNA strand displacement for mutations in the interested site of the TSHR gene. SSCP results of these samples are shown in Fig. 1. Samples that revealed DNA strand displacements by SSCP analysis were directly sequenced and mutations in the TSHR gene were identified in 10 of 58 hyperfunctioning thyroid nodules (Fig. 2). All of them had been described previously and were found to be gain-of-function mutations producing a stimulation of cAMP. All the mutations identified in this study were located in the sixth transmembrane segment of the TSH receptor, except the mutation located at the amino acid position 623 in the third intracellular loop. Clinical findings for patients with somatic TSH receptor mutations are shown in Table 2.

Seven (20%) of the patients had never lived in an endemic goiter region. On the other hand 28 (80%) patients had lived in endemic goiter regions during their lifetime. In the present study 10 (17%) TSHR mutations were found in hyperactive thyroid nodules. Nine (90%) patients with mutations had lived in endemic goiter regions, whereas only one patient (10%) with a mutation lived in an iodine repleted area of Turkey (Table 3). But statistical analysis has not shown a direct relationship between mutation positivity and residence in the endemic region ($p = 0.64$)

Discussion

In the present study we screened for activating mutations in exon 8–9 of the $G_S\alpha$ gene and PKA Cα at Trp 196 Arg in hyperactive thyroid nodules by SSCP analysis, but could not detect any.

TSHR mutations in thyroid nodules was first reported by Parma *et al.* They found mutations in 3 (27%) of 11 functional thyroid adenomas [36]. In two other studies, TSHR mutations were detected in 27 (82%) of

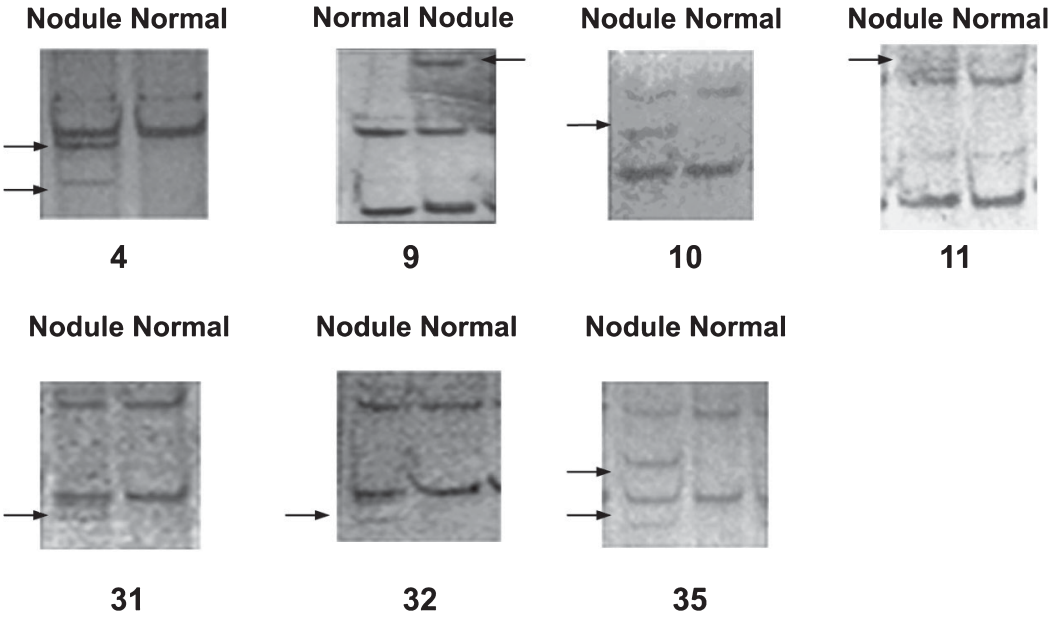

Fig. 1. SSCP gel analysis patterns of the PCR products (codon 605–664 and 482–619 of the TSH receptor); showing abnormal migration patterns (Arrows show mutant bands).

Table 2. Clinical findings for patients with somatic TSH receptor mutations

No	Gender	Age (year)	Duration of Goiter (year or month)	Thyroid function	Living in endemic goiter region (+/–)	Nodule location	Nodule size (cm), Macroscopically	Mutation
4	M	52	18y	Hyperthyroid	+	Isthmus	3 × 3	Leu 629 Phe (ttg→ttt)
9	F	39	18y	Euthyroid	–	Right lobe, central	1 × 1	Ala 623 Val (gcc→gtc)
10	M	20	2y	Hyperthyroid	+	Right lobe, central	5 × 4	Leu 629 Phe (ttg→ttc)
11	F	53	30y	Hyperthyroid	+	Left lobe, inferior pole	4 × 3.5	Asp 633 His (gac→cac)
29	F	50	4y	Hyperthyroid	+	Right lobe, inferior pole	1 × 1	Ile 630 Leu (atc→ctc)
31	M	50	5m	Hyperthyroid	+	Right lobe, central	5 × 5	Ile 630 Leu (atc→ctc)
32	F	28	8m	Hyperthyroid	+	Left lobe, central	3 × 4	Ile 630 Leu (atc→ctc)
33	F	18	6y	Hyperthyroid	+	Right lobe, inferior pole	5 × 4	Asp 633 Tyr (gac→tac)
34	M	58	2y	Hyperthyroid	+	Left lobe, inferior pole	5 × 3.5	Thr 632 Ile (acc→atc)
35	F	30	17y	Euthyroid	+	Left lobe, inferior pole	2 × 2	Leu 629 Phe (ttg→ttc)

Table 3. Comparison of mutation positivity and residence in the endemic region

	Living in endemic goiter regions		
	Never	Ever	Total
Mutation (–)	1 (10%)	9 (90%)	10 (100%)
Mutation (+)	6 (24%)	19 (76%)	25 (100%)
Total	7 (20%)	28 (80%)	35 (100%)

$p = 0.64$

33 functional thyroid adenomas from Belgium [37] and in 8 (72%) of 11 toxic adenomas in addition to 5 (83%) of 6 toxic multinodular goiters from Italy [22] by direct sequencing analysis. The study, in which the frequency of TSHR mutation was the highest, was reported by Nogueira from Brazil. TSHR mutations were identified in 6 (86%) of 7 functional thyroid adenomas by direct sequencing analysis [14]. Studies screening a higher number of samples identified 43 (57%) mutations in 75 toxic nodules [11] and in 15 (48%) of 31 toxic thyroid nodules [17], respectively. On the other hand, TSHR mutations were either seldom reported or not detected in other studies [13, 21, 25, 38, 39].

It has been proposed that differences in iodine intake could account for the variable prevalence of TSHR mutations [14]. Activating mutations in the cAMP signal transduction pathway are related to iodine intake. Iodine deficiency increases serum TSH levels and increases sensitivity of the thyroid follicular cells to TSH, increasing the mitotic activity of thyroid follicular cells in return, thus the possibility of mutagenesis [40]. Goiter prevalence in Turkey was reported as high as 30.5% in 1988 [27] and as 31.8% in 2002 [26]. The present study depicts that the majority of patients with goiter (80%) and mutation positivity (90%) have spent their lives in the endemic goiter regions but statistical analysis has not shown a direct relationship between mutation positivity and residence in the endemic region. This finding may be related to the heterogenicity between the groups living in endemic and non-endemic regions.

The incidence of TSHR mutations was found to be 17% in this study.

The low incidence of somatic TSHR mutations might be caused by several reasons. Firstly, using sensitive techniques is of prime importance. We used SSCP analysis to examine TSHR genetic alterations in thyroid nodules. This might be the reason why the frequency of TSHR mutation was low in our study. Denaturing gradient gel electrophoresis (DGGE) is the most sensitive technique in detection of TSHR genetic alterations [41]. Detection of heterozygous somatic muta-

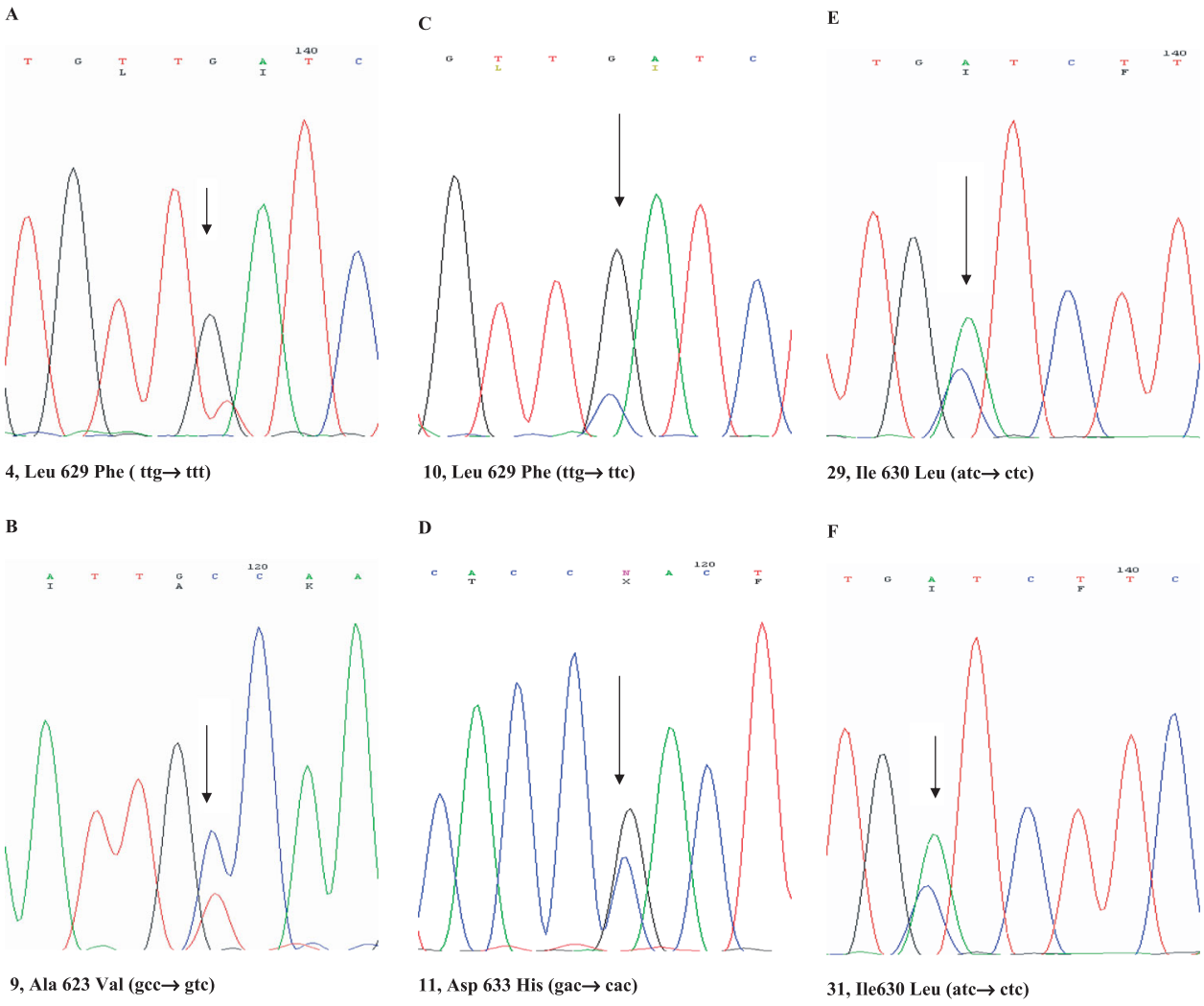

Fig. 2. Direct sequencing analysis results of PCR products from the nodules in patients
A. no: 4, B. no: 9, C. no: 10, D. no: 11, E. no:29, F. no: 31, G. no: 32, H. no:33, I. no: 34, J. no: 35.

tions by direct sequencing requires a ratio of mutated to wild type alleles close to 1:1. But normally DNA samples from tissues obtained at surgery rarely display this ratio due to contamination with blood and stromal cells and the possible presence of degenerate areas within the nodules. Therefore, detection of mutant alleles by direct sequencing or SSCP analysis is less sensitive [11]. In a previous study from Japan, 45 autonomously functioning thyroid nodules were investigated for the presence of activating TSHR mutations by SSCP and no mutations were found [25]. In some studies in which direct sequencing was used, the incidence of TSHR mutations was also low. In one study, only 3 (8%) of 37 toxic thyroid nodules(TTNs) harbored TSHR mutations [13]. In two other studies, the frequencies of TSHR mutation in TTNs were reported as 22% (2 of 9) [38] and 27% (3 of 11) [36], respectively. On the other hand DGGE identified 43 mutations (57%) in 75 toxic thyroid nodules in another study [11].

Secondly, in our study only a part of exon 10 of TSHR was analyzed. When the entire exon 10 of the TSHR was sequenced, the frequency of TSHR mutation was found to be high [5, 6, 11, 14, 16, 17, 22, 37], whereas the studies in which only parts of the TSHR were examined, reported low frequencies of TSHR mutations [13, 25, 38]. Therefore, if the entire exon 10 of TSHR had been screened, the frequency of mutation could have been found to be higher.

But on the other hand, speculations on the molecular

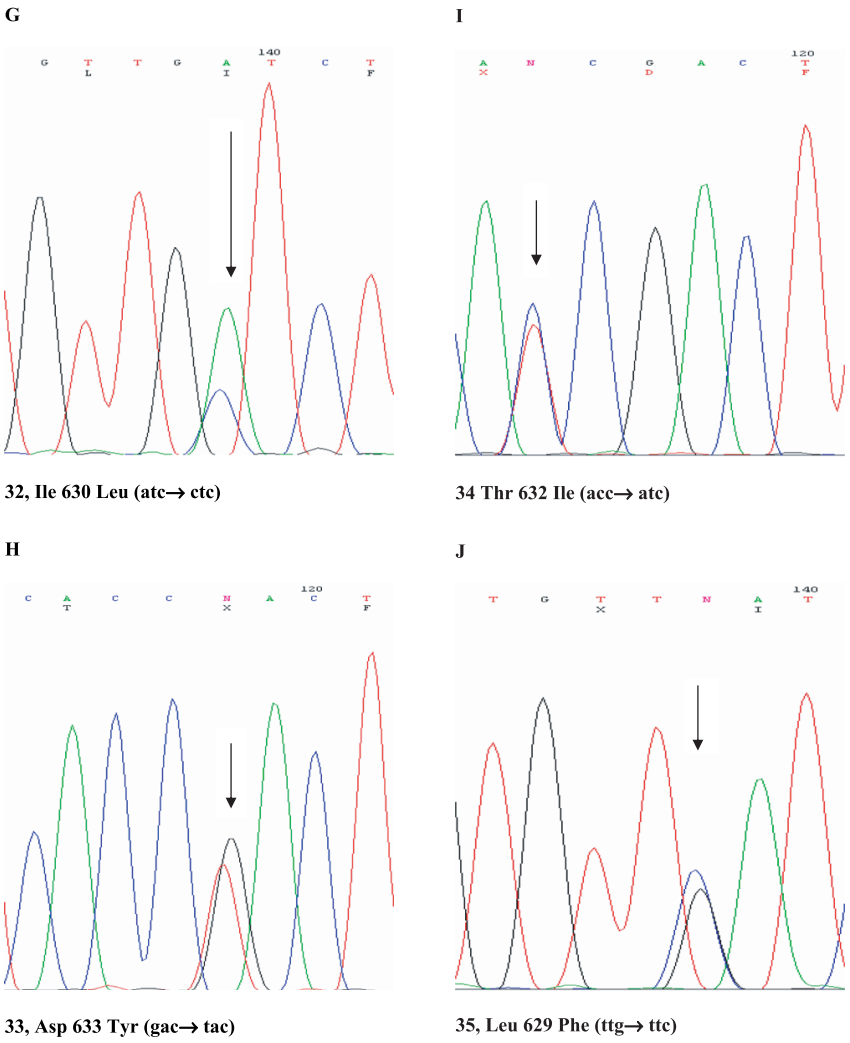

Fig. 2. (continued)

etiology of mutation-negative toxic thyroid nodules should constitute another important topic of debate. The clonal origin of thyroid tissue is an important issue. Mutation negative-nodules could contain polyclonal lesions. But 50% of mutation negative-thyroid nodules show a monoclonal origin when tested for X chromosome activation, so there might be other theories of the molecular etiology of mutation negative-thyroid nodules. Mutation in other candidate genes of the cAMP signal transduction pathway (*e.g.*, other G-protein subunits, adenyl cyclase, phosphodiesterase) or molecules activating other signaling cascades (*e.g.*, tyrosine kinase cascade through IGF-1) might cause toxic TTNs. Further, overexpression of signaling proteins such as TSHR, $G_s\alpha$ and adenyl cyclase might cause toxic adenoma or toxic MNG. Especially, expression profiles of the other signal proteins by microarray method could provide new insights into the etiology of toxic adenoma and toxic MNG [19, 40, 42]. Thus, the etiology of mutation-negative toxic thyroid nodules in Turkey should be sought taking all of these theories into consideration.

Forty-three activating TSHR mutations (29 somatic and 19 germline) have been reported since then. With the exception of residue 183 and residue 281, gain-of-function TSHR mutations are exclusively located in the transmembrane spanning region [35]. In our study, one mutation was located at amino acid position 623 in the 3rd intracellular loop. All other mutations found in this study were located in the 6th transmembrane segment

of the TSHR, confirming this region as a hot spot area for TSHR mutations. In accordance with the literature, all mutations were somatic and heterozygotic.

In this study, the frequency of mutations in the TSHR signal transduction pathway were found to be lower than expected in our country. Further research is needed using more sensitive techniques. In addition, other exons of the TSHR should also have been investigated for somatic mutations. Moreover, other steps in the cAMP signal transduction pathway or other signal transduction pathways should also be investigated in the pathogenesis of autonomously functioning thyroid nodules.

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