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HEAD AND NECK

United detection GNAS and TSHR mutations in subclinical toxic multinodular goiter

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Abstract The aim of the study was to investigate whether the mutations of the GNAS gene and thyroid-stimulating hormone receptor (TSHR) gene were a potential molecular biological mechanism for subclinical toxic multinodular goiter (sTMG) and to evaluate the association of these mutations with the clinicopathological features of these disorders. Forty-four patients with sTMG and 20 controls (multinodular goiter) from Heilongjiang province of China who underwent subtotal thyroidectomy were recruited. Genes' mutations were analyzed by direct DNA sequencing of the polymerase chain reaction-amplified the parts of exons. In sTMG group, three mutations at GNAS gene were identified in seven patients (15.9%). Six mutations at TSHR gene were identified in 14 patients (31.8%). Mutation positivity of TSHR gene had statistically significant by comparison of two groups. In sTMG group, the mutation positivity of patients with serum TSH level below 0.1 μU/ml and above 0.01 μU/ml is obviously different ($P < 0.05$) at TSHR gene. However, these statistically significant differences were both not being seen at GNAS gene, and patients with nodules before universal salt iodization (USI) and after USI ($P > 0.05$). Mutation positivity of TSHR gene has a relation with sTMG. It is more probable that serum TSH level play an important role in mutagenesis.

Keywords TSHR gene · GNAS gene · Subclinical toxic multinodular goiter · Mutations

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Introduction

Subclinical hyperthyroidism, defined by normal circulating levels of free T_4 and T_3 and low levels of thyroid-stimulating hormone (TSH) [1], was a common clinical entity and was typically caused by the same conditions that account for the majority of cases of overt hyperthyroidism: Graves' disease, toxic multinodular goiter (TMG), and solitary autonomously functioning thyroid nodules. The epidemiology of subclinical hyperthyroidism showed that among these effective factors, regional iodine intake deficiency had been shown to be associated with the prevalence [2]. Subclinical TMG(sTMG)was a condition in which the thyroid gland contains multiple lumps (nodules) that were normal circulating levels of free T_4 and T_3 and low levels of TSH. It might be a transition from multinodular goiter (MG) to TMG [3]. Subclinical hyperthyroidism was an increasingly recognized disease. However, the pathogenesis and etiology of this disease was still unclear and there were only a few literature reports.

Thyroid-stimulating hormone is the main trophic factor that controls thyroid function and growth. TSH binds to its receptor (TSHR) and activates the α-subunit of the stimulatory G protein (Gsα), leading to adenylate cyclase (AC) activation and subsequently cyclic AMP (cAMP) production.

In the last few years, several studies described mutations in thyroid hormone pathway genes such as the TSH receptor (TSHR) gene and GNAS (guanine nucleotide-binding protein, alpha stimulating) gene that causing permanent activation of the thyroid follicular cell AC, had been shown to be the most probable cause of the hyperfunction and growth of toxic adenoma [4–7]. In contrast to the hyperthyroidism, the investigation of the genes in subclinical hyperthyroidism was reportedly less in forepassed literatures.

In Heilongjiang province of China, the incidence of MG is high because of iodine deficiency (20%) [8]. In the present study, we had selected 44 patients with sTMG and 20 euthyroid patients with MG and examined genetic variation in GNAS subunits gene and TSHR gene of their thyroid nodular tissues to determine mutations. The sequence of the exons 8–10 is well conserved in GNAS gene, which encodes adenylyl cyclase interaction site. Previous investigations suggested amino acid residues coded the exons 1–9 of TSHR gene were particularly binding sites of thyroid stimulatory antibodies and thyroid stimulatory hormone in extracellular domain. Therefore, we focused on exons 8–10 of the GNAS gene and exons 1–9 of TSHR gene. The aim was to screen the connection between genetic variations and pathogenesis of sTMG. The study was approved by the local ethics committee, and informed consent was obtained from all the participants prior to testing.

Materials and methods

Clinical and laboratory examination

Between March 2006 and February 2008, 64 patients (44 patients with sTMG and 20 patients with MG) who underwent subtotal thyroidectomy were reviewed at the Department of Surgery of the First Affiliated Hospital of Harbin Medical University. These patients with MG did not receive any suppressive medicamentous therapy before surgery. Subtotal thyroidectomy was to relax compressive symptoms resulting from the MGs which were suggested benign lesions by preoperative ultrasound-guided fine-needle aspiration (US-FNA). The average age was 43.9 ± 8.76 years (range 28–57 years) and included 52 females and 12 males.

Every patient underwent radioimmunology analysis of serum TSH, FT_3 and FT_4 twice (interval 7–14 days) before surgery. According to the results of thyroid function tests, the patients were divided into sTMG group ($n = 44$) and MG group (control group $n = 20$). The nodular US-FNA underwent by three experienced operators using a 22-gauge needle attached to a 10-ml syringe at the Department of Ultrasound of the First Affiliated Hospital of Harbin Medical University. The IU22 ultrasound scanner (Philips Medical Systems, NA, Bothell, WA, USA) was used.

All patients came from Heilongjiang province and their birthplace and habitation were recorded. These patients were excluded because of other clinical settings: central hypothyroidism, physiological lowering of serum TSH at the end of the first trimester of pregnancy, and low-serum TSH levels in the critically ill and elderly people. Some characteristics of the patients and their adenomas were summarized in Table 1.

Tissue specimens

Optical and electron microscopic observations were performed for all specimens. The pathological features of sTMG consist of follicles of different size and heterogeneity. Some epitheliums of functional inaction become flat, but other epitheliums of functional activity become columnar. Electron microscopic observation showed that many changes appear in columnar epitheliums, including dense microvillus, abundant rough endoplasmic reticulum, abundant elliptic colloids and obvious nucleoli. The pathological features of MG are a part of follicles excess dilation and epitheliums of MG become flat. Electron microscopic observation showed flat epithelium with thin microvillus and underdeveloped rough endoplasmic reticulum and Golgi body.

The preoperative cytopathological slides of US-FNA and post surgery histopathological slides were reviewed by three pathologists. Nodular tissues and adjacent normal thyroid tissues were obtained by the results of thyroid histopathological examination as a guide from 64 unrelated patients. One hundred and twenty-eight fresh samples were stored at −80°C in the refrigerator.

Table 1 Patients clinical data

	sTMG	MG	Normal values
No.	1–44	45–64	
Gender			
Female	36 (81.8%)	16 (80.0%)	
Male	8 (18.2%)	4 (20.0%)	
Age (years)	28–57	30–57	
Duration of goiter			
>12 years	12 (27.2%)	7 (35.0%)	
≤12 years	32 (72.8%)	13 (65.0%)	
TSH (μU/ml)			
0.1–0.2	24 (54.5%)		0.27–4.2
<0.1	20 (45.5%)		
FT_3 (pg/ml)			1.8–4.6
FT_4 (ng/dl)			0.93–1.7

DNA isolation

30–40 mg frozen tissues of 128 samples were triturated in liquid nitrogen by a mortar. Genomic deoxyribonucleic acid (DNA) was isolated from trituration tissues using DP-304 Extraction DNA from Blood/Tissue/Cell Kit (TIANGEN of Beijing, China) according to the descriptive steps of the Kit. Genomic DNA was re-suspended in 50 µl TE pH 7.5 (10 mM Tris–HCl, 1 mM EDTA). Consistence was measured by Amersham-Biosciences GeneQUANT Pro and stored at −20°C in the refrigerator.

Polymerase chain reaction

Polymerase chain reaction (PCR) amplify exons 8–10 of the GNAS subunits gene and exons 1–9 of TSHR gene (encoding extracellular domain of TSHR) and were performed by the Biometra T Gradient Thermocycler (Biometra, Gottingen, UK). 50 µl of reaction mixture system contains 0.5 µg of genomic DNA, 10 pmol of each primer (primers sequence in Table 2, were synthesized by Shanghai Sangon, China), 25 µl of MasterMix (KP201-Pfu PCR MasterMix Kit, Tiangen of Beijing, China) and some ddH_2O for a final 50 µl volume. Samples were denatured at 95°C for 10 min, followed by 35 cycles of amplification. Each cycle consisted of denaturation at 95°C for 30 s, primer annealing for 30 s (temperature in Table 2), and primer extension at 72°C for 30 s, with a final additional extension step at 72°C for 4 min. The amplified products were electrophoresed in 1.2% agarose gel and their results analyzed by gel system of Tanon Gis-2020.

Sequence analysis

The amplification products were purified and sequenced on both strands by sequence analysis at the Department of Shanghai Sangon. Samples were sequenced on ABI PRISM 3730, and the reagent used was BigDye terminator v3.1.

Statistical analysis

All data were expressed as mean ± standard deviation. χ^2 test was used to compare the mutation positivity of TSHR and Gsa gene between sTMG and MG group. Differences were considered significant at $P < 0.05$. The SPSS version 10.0 (SPSS, Chicago, USA) was used for statistical analysis.

Results

By sequencing PCR products, in sTMG group, three mutations (A1176 deletion, A1188G and A1191 deletion) of the exon 10 of GNAS gene were identified in 7 of 44 (15.9%) patients (No. 7, 12, 18, 21, 26, 29, 40) with sTMG at codon 274, 278, 279 (Table 3; Fig. 1). Six mutations of the TSHR gene were identified in 14 of 44 (31.8%) patients (No. 4, 8, 13, 15, 16, 19, 21, 25, 27, 29, 33, 35, 40, 42) with sTMG, including C250A, C228G, T insertion at 222–223, T insertion at 268–269, T224A and C276A (Table 4; Fig. 2). In addition, in MG group, no mutations were found at GNAS gene and one mutation (A insertion at 331–332) were identified at TSHR gene. All mutations were detectable only in the nodular tissue, not in the surrounding tissue. In the sTMG group, the mutation positivity of GNAS and TSHR gene was significantly higher than the MG group and had statistical significant ($P < 0.05$) at TSHR gene, but which was not being seen at GNAS gene ($P > 0.05$) (Table 5).

In sTMG group, by comparing mutation positivity between patients with different serum TSH level, we found 20 patients with serum TSH level below 0.1 µU/ml had six mutations at GNAS gene (30.0%)and 10 mutations at TSHR gene (50.0%), 24 patients with serum TSH level equal or above 0.1 µU/ml had one mutations at GNAS gene (4.2%)and four mutations at TSHR gene (16.7%). However, the statistical analysis indicated that there is no statistically significant difference at ($P > 0.05$) in GNAS gene, but which was being seen in TSHR gene ($P < 0.05$) (Table 6).

Table 2 Primers sequence used for the PCR amplification of the TSHR and GNAS

Exon	Forward primer	Reverse primer	Annealing temperature (°C)
TSHR1	5CGATTTCGGAGGATGGAG3	5TTCGGGCTGTTATTGAGC3	56
TSHR2	5CAGCCAACATATTGTGAA3	5TCATGTAGGAATTAGGGA3	46
TSHR3	5GAATCCATGAGGGTTGTA3	5AGTGGTTGTGGCTTAGGT3	54
TSHR4	5CCCGTGAGGAGACAGGAGT3	5GTGCTGGACATTTGAGTGC3	60
TSHR5	5GTTGTCTTCAGAACCCAT3	5GTCCTTGCCTTACTTCAT3	47
TSHR6	5AGACCTCTGAGCCACTAG3	5ACCATCCCATAAGACAAT3	47
TSHR7	5ATGGGCATTCAAACTGAG3	5TAGGGTAAGGGAAAGTGA3	50
TSHR8, 9	5CTCACCACCCTCACCATC3	5TTCTTCCTTCTTTGTGCC3	52
GNAS8, 9, 10	5CGTGCTGTGCTGTTTGTG3	5TCCTTTATGGTTTGGTGG3	57

Primers designed by Technology Department of Shanghai Sangon

Table 3 Results of GNAS mutation

No.	Gender	Age (years)	Duration of goiter (years or months)	TSH (μU/ml)	GNAS mutations
7	F	28	8 months	0.09	A1188G
12	M	37	14 years	0.06	A1176deletion
18	F	43	1 year	0.09	A1188G
21	M	53	16 years	0.08	A1191deletion
26	F	48	18 years	0.06	A1191deletion
29	F	35	5 years	0.03	A1176deletion
40	F	41	8 years	0.11	A1188G

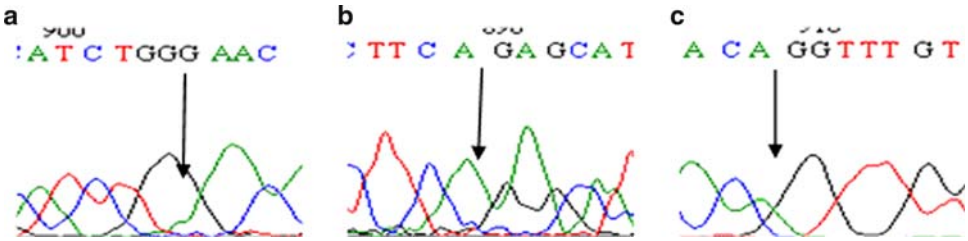

Fig. 1 Mutation results of GNAS gene in patients. **a** No. 7, 18, 40: A1188G, **b** No. 12, 29: A1176 deletion, **c**: No. 21, 26: A1191 deletion

Table 4 Results of TSHR mutation

No.	Gender	Age (years)	Duration of goiter (years)	TSH (μU/ml)	GNAS mutations
4	F	51	15	0.06	C250A
8	F	48	13	0.03	C228G
13	F	48	6	0.16	T insertion at 222–223
15	F	40	4	0.04	T insertion at 268–269
16	F	33	3	0.09	T224A
19	M	56	15	0.10	C250A
21	M	53	16	0.08	T insertion at 222–223
24	F	37	7	0.05	C276A
27	F	33	1	0.17	T insertion at 222–223
29	F	35	5	0.03	T224A
33	F	52	3	0.01	C250A
35	F	59	14	0.07	C276A
40	M	44	6	0.04	T341 deletion
42	F	42	8	0.11	A insertion at 331–332
60	F	46	7	2.12	A insertion at 331–332

In addition, in sTMG group, 12 patients with nodules (duration of goiter ≥12 years) before universal salt iodization (USI) were detected with three mutations (25.0%) at GNAS gene and five mutations with TSHR gene (41.7%). In addition, 32 patients with nodules after USI were detected with four mutations at GNAS gene (12.5%) and nine mutations with TSHR gene (28.1%); however, there is no statistical significant difference (Table 7).

Discussion

Heilongjiang province of China is iodine-deficient area and the incidence of MG is high (20%). Subclinical hyperthyroidism was more prevalent in some current and previous iodine-deficient areas. It was established that long-term iodine deficiency might lead to hyperfunction, which was one of the causes of subclinical hyperthyroidism [2, 9]. Since 1995, the incidence of TMG had an increasing trend following the adoption of USI. The pathogenesis of TMG and sTMG still remains to be unclear. Previous studies indicated that sTMG might be a transition form from MG to TMG, whereas the mechanism of this process was also not clear.

The etiology of subclinical hyperthyroidism was divided into two categories: exogenous and endogenous. Exogenous subclinical hyperthyroidism was due to the ingestion of intentional or unintentional suppressive doses of thyroid

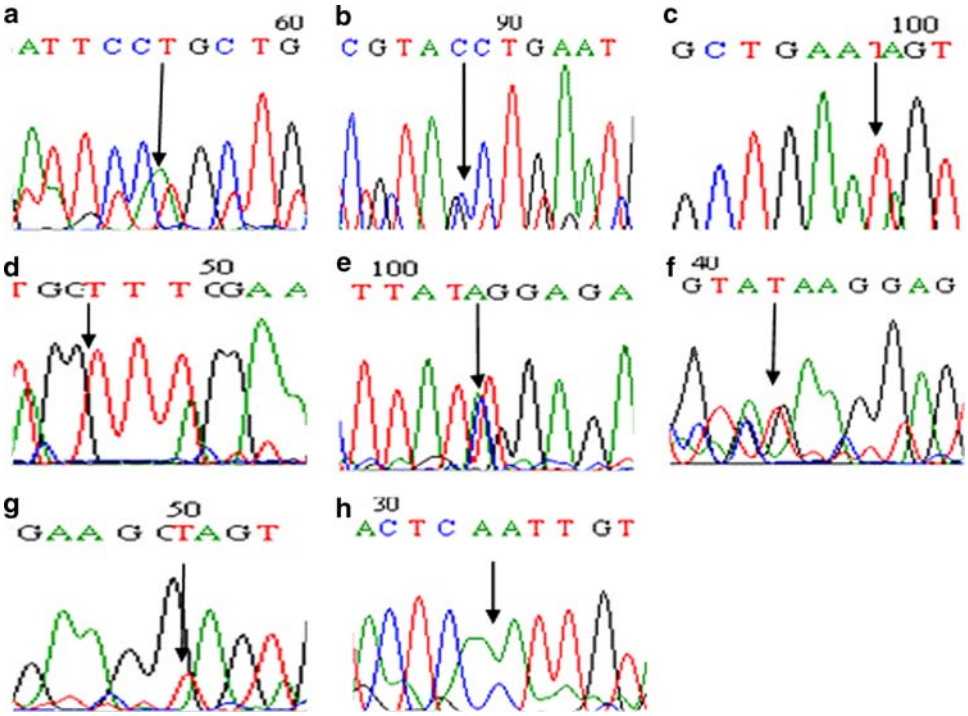

Fig. 2 Mutation results of TSHR gene in patients. **a** No. 4, 19, 33: C250A (antisense sequence), **b** No. 8: C228G (antisense sequence), **c**: No. 13, 21, 27: T insertion at 222–223, **d** No. 15: T insertion at 268–269 (antisense sequence), **e** No. 16, 29: T224A, **f** No. 24, 35: C276A (antisense sequence), **g** No. 42, 60: A insertion at 331–332, **h** No. 40: T343 deletion (antisense sequence)

Table 5 Comparison of mutation positivity between sTMG and MG

	GNAS		Total	TSHR		Total
	Mutation (+)	Mutation (−)		Mutation (+)	Mutation (−)	
sTMG	7	37	44	14	30	34
MG	0	20	20	1	19	20
χ^2			2.13			5.33
P			>0.05			<0.05

Table 6 Comparison of mutation positivity between patients with serum TSH level below 0.1 μU/ml and above 0.1 μU/ml in sTMG group

serum TSH (μU/ml)	GNAS		Total	TSHR		Total
	Mutation (+)	Mutation (−)		Mutation (+)	Mutation (−)	
≤0.1	6	14	20	10	10	20
>0.1	1	23	24	4	20	24
χ^2			3.68			4.16
P			>0.05			<0.05

Table 7 Comparison of mutation positivity between patients with nodules before and after USI in sTMG group

	GNAS		Total	TSHR		Total
	Mutation (+)	Mutation (−)		Mutation (+)	Mutation (−)	
USI						
Before	3	9	12	5	7	12
After	4	28	32	9	23	32
χ^2			0.299			0.246
P			>0.05			>0.05

hormone, and endogenous subclinical hyperthyroidism was resulted from some thyroid diseases, such as Graves' disease, TMG, autonomously functioning solitary nodules, and various forms of thyroiditis. The risk of progression to overt hyperthyroidism was lower (0.9–4.1%) for patients with subclinical hyperthyroidism and a majority of cases

may frequently resolved spontaneously. However, the etiology of subclinical hyperthyroidism might play a role in determining whether subclinical hyperthyroidism resolves or progresses. For example, Woeber [10] observed that five of seven patients with Graves' disease and subclinical hyperthyroidism (baseline TSH 0.03–0.06 mU/l) when followed up for 3–19 months, whereas it remained subnormal (baseline TSH 0.1–0.29 mU/l) in patients with MGs when followed up for 11–36 months. In this study, we selected subclinical hyperthyroidism resulted from MGs. Patients with large nodular thyroids and above normal serum TSH concentrations may be at a particular risk for developing overt hyperthyroidism when exposed to higher concentrations of iodine [11]. In thyroid, the cAMP pathway plays a key role in mediating the effects of TSH on thyrotroph cell growth and function. In the last decade, studies were first done to determine the frequency of GNAS and later TSHR mutations in TMG. Different frequencies, ranging from 0 to 38% for GNAS mutations and from 3 to 86% for TSHR mutations, were found [12]. In addition, exons 8, 9, 10 of the GNAS and the exon 10 of the TSHR gene were always the hot-spot sections in the earlier studies. There were limited case reports related to GNAS and TSHR genetic alterations in subclinical multinodular hyperthyroidism.

In the present study, we examined TSHR gene and GNAS gene mutations in 64 patients from Heilongjiang of China. We screened the hot-spot exons 8, 9, 10 of the GNAS and exons 1–9 of the TSHR gene coding the extracellular domain. Three mutations (A1176 deletion, A1188G and A1191deletion) at the exon 10 of GNAS gene were identified in 7 of 44 patients (No. 7, 12, 18, 21, 26, 29, 40) with sTMG (15.9%) at codon 274, 278, 279 of gene. Six mutations at the TSHR gene were identified in 14 of 44 (31.8%) patients (No. 4, 8, 13, 15, 16, 19, 21, 25, 27, 29, 33, 35, 40, 42) with sTMG, including C250A, C228G, T insertion at 222–223, T insertion at 2268–269, T224A and C276A. In MG group, no mutations were found at GNAS gene and one mutation (A insertion at 331–332) was identified at TSHR gene. In MG group, none mutation was detected in GNAS gene, one mutation was identified at TSHR gene. We did not determine the functional consequences of these mutations in vitro. However, mutations might cause abnormal splicing. The sequence of the exons 8–10 is well conserved in GNAS gene, which encodes AC interaction site. Previous investigations suggested amino acid residues coded exons 1–9 of TSHR gene were particularly the binding sites of thyroid stimulatory antibodies and thyroid stimulatory hormone in extracellular domain. Thus, these mutations can cause abnormal protein sequence. As a result, the function of proteins possibly changes.

In the sTMG group, the mutation positivity of GNAS and TSHR gene was significantly higher than the MG group. Statistical analysis indicated significant difference in the mutation rate of TSHR gene between sTMG and MG group which was not observed at GNAS gene. This result may be related with sample size.

Our study showed, in sTMG group, that 20 patients with serum TSH level below 0.1 μU/ml had six mutations at GNAS gene (30.0%) and ten mutations at TSHR gene (50.0%), 24 patients with serum TSH level equal or above 0.1 μU/ml had one mutation at GNAS gene (4.2%) and four mutations at TSHR gene (16.7%). The mutation positivity of GNAS and TSHR gene was significantly higher in cases of serum TSH level below 0.1 μU/ml than those of above 0.1 μU/ml. This results implied that sTMG may be related with iodine deficiency which increases serum TSH levels, sensitivity of the thyroid follicular cells to TSH and elevates the mitosis of thyroid follicular cells resulting in the gene mutations [13–15]. These mutated cells formed autonomously functioning cellular mass without enough size increasing synthetic ability of hormone, thus decreasing serum TSH levels, and to maintain normal serum T_3 and T_4 levels by restraining the normal thyroid follicular cells. We found different size and heterogeneity follicles on the pathological slides of sTMG, including epitheliums of functional inaction (flattening) and epitheliums of functional activity (columnar).

In 1995, the strategies for the prevention and control of iodine deficiency disorders were realized in Heilongjiang; and thus for USI, supplementation were introduced. USI had been agreed to be an effective means in eliminating iodine deficiency. However, after the increment in iodine intake, an increasing incidence of hyperthyroidism due to the occurrence of iodine-induced hyperthyroidism was observed in many previously iodine-deficient areas [16–18]. In our cases, 12 patients with nodules (duration of goiter $\geq$12 years) before USI were detected with three mutations(25.0%) at GNAS gene and five mutations at TSHR gene (41.7%). In addition, 32 patients with nodules after USI were detected four mutations at GNAS gene (12.5%) and nine mutations at TSHR gene (28.1%). We found no statistical significant difference when comparing mutation positivity between patients with nodules before USI and after USI in sTMG group; this results implied that USI had no obvious influence on changes of GNAS and TSHR gene.

In this study, we investigated the frequency of mutations of the GNAS and TSHR gene and found mutation positivity of TSHR gene had a relation with subclinical TMG. In addition, serum TSH level might play an important role in mutagenesis. However, further research is needed to determine the functional consequences of these mutations in vitro. In addition, other exons of GNAS gene and exon 10 of TSHR gene and outcome of sTMG should also be investigated in the subsequent investigation for more clarifying connection between the pathogenesis of sTMG with changes of GNAS gene and TSHR gene.

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