DETECTION OF HOMOZYGOUS G.IVS5+1G>A, G.IVS34-1G>C AND C.886C>T MUTATIONS AMONG TOXIC GOITER AND THYROID CANCER PATIENTS IN IRAQ

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ABSTRACT

Forty eight DNA samples from 31 toxic goiter patients and 17 thyroid cancer patients were analyzed to detect g.IVS5+1G>A, g.IVS34-1G>C and c.886C>T mutations of the thyroglobulin (TG) gene. Among these samples three homozygous mutations were detected. Two of these mutations were detected in two toxic goiter patients as guanine to adenine transition g.IVS5+1G>A at position +1 of the donor splice acceptor site in exon-intron 5 and transversion that replaced guanine by cysteine (g.IVS34-1G>C) in the exon 34. The third homozygous mutation was detected among one thyroid cancer patient as transition that replaced cysteine by thymine c.886C>T in the exon 7. No homozygous g.IVS34-1G>C mutation was detected in thyroid disorders before, to our knowledge this is the first time by which homozygous mutation was detected in one toxic goiter patient.

Key wards: Thyroid disorders, TG gene, g.IVS5+1G>A, g.IVS34-1G>C, c.886C>T.

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تعقب الطفرات الوراثية متجانسة الزيجة g.IVS5+1G>A, g.IVS34-1G>C, c.886C>T في مرضى تضخم الغدة الدرقية السام و سرطان الغدة الدرقية في العراق

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الخلاصة

حللت 48 عينة DNA تعود الى 31 مريض بتضخم غدة الدرقية السام و17 مريض بسرطان الغدة الدرقية من أجل تعقب الطفرات الوراثية g.IVS5+1G>A, g.IVS34-1G>C, c.886C>T لمورث الثايروجلوبيولين Thyroglobulin (TG). وجد من خلال التحليل إن هناك ثلاثة طفرات وراثية متجانسة الزيجة Homozygous . إثنان منها تم تعقبها في مريضين بتضخم غدة الدرقية السام و كانت الطفرات عبارة عن إستبدال مكافئ للجوانين بالادنين (g.IVS5+1G>A) في الموقع +1 لفاصل المحور 5 و إستبدال غير مكافئ للجوانين بالسايتوسين (g.IVS34-1G>C) في المحور 34 . أما الطفرة الثالثة متجانسة الزيجة المسجلة فقد تم تعقبها في مريض بسرطان الغدة الدرقية و كانت عبارة عن إستبدال مكافئ للسايتوسين بالثايمين في المحور 7 (c.886C>T). لم تسجل سابقاً الطفرة الوراثية (g.IVS34-1G>C) كطفرة متجانسة الزيجة عند مرضى الغدة الدرقية و سجلت هنا لأول مرة عند مريض واحد مصاب بتضخم الغدة الدرقية السام .

INTRODUCTION

Thyroglobulin (TG) gene is a key element in thyroid hormones synthesis and production. It codes thyroglobulin protein, a large homodimeric protein with a molecular weight of 330 KDa per monomer which iodinated by thyroid peroxidase (TPO) via forming thyroxine (T4) and triiodothyronine (T3) in process done by thyroid follicular cells (1,2).The failure of this process led to a variant type of thyroid disorders (3). Most of this failure due to mutations occurred either in TG genes or TPO gene. The most frequent mutations detected were heterozygous mutations in TG gene. A rare homozygous mutations of the TG gene were recorded among thyroid disorders until now(OMIM,http://www.ncbi.nlm.nih.gov/omim/188450). These rare mutations include a homozygous guanine to adenine point mutation at position +1 of the splice donor site of intron 5 (g.IVS5+1G>A) was detected in a patient with recurrent goiter and a metastatic follicular thyroid carcinoma (4). A homozygous c.886C>T mutation in exon 7 which result in a premature stop codon at amino acid 277 (p.R277X) was also detected in goiter patients with congenital hypothyroidism (5). On the other hand, no homozygous g.IVS34-1G>C mutation is detected in thyroid disorders yet. Other TG homozygous mutations such as g.IVS30+1G>T, p.Q2142X, p.C1245R, p.C1977S and p.C1058R mutations were identified in patients with various thyroid disease or without thyroid disorder (6,7,8). The present work aimed to detect the frequency of TG homozygous mutations among thyroid disorder patients.

MATERIAL AND METHODS

Subjects

Forty eight patients (18 males and 30 females; 31 with toxic goiter and 17 with thyroid cancer) as well to twenty-five (12 male and 13 female) healthy individuals as control were analyzed. The patients age ranging from 30 to 79 years. The patients were attended the endocrinologist in Nuclear Medicine Hospital and Al Yarmok Nuclear Medicine Department in Baghdad-Iraq during a period from July 2009 to October 2009.

Isolation of genomic DNA

Three ml venous blood was collected in EDTA tubes and genomic DNA was extracted according to DNA extraction Kit protocol from Promega-Canada. The DNA concentration and purity were estimated (9).

Locked Nucleic Acid (LNA)-primers-PCR

LNA-primer PCR was carried out according to (10),(11) and (12). Three TG common mutations were selected in this study table)1). The mutations primers were designed and identified using NCBI tools. These mutations include g.IVS5+1G>A, g.IVS34-1G>C and c.886C>T located in exons 5,34 and 10 of TG, respectively.

The modified LNA-primers were used for the detection of TG gene mutations. The design of these primers sequence were done using GeneFisher and Oligoanalyzer (https://www.idtdna.com/analyzer/Applications OligoAnalyzer; http://lnatools.com; http://biowww.net /protocols/index.php). and primer BLAST programs. Degenerated primers can be designed depending on gene sequences homology. GeneFisher and Oligoanalyzer are an interaction web-based programs for designed degenerated primers.

Table(1):LNA- primer sequences and LNA base modification used for PCR amplification of *TG* gene.

Mutation	LNA Primer Foreward (5‘→3‘)	LNA Primer Reverse (5‘→3‘)
	TG gene	
	Exon 5/Intron	
g.IVS5+1 G>A	FW-*TG*- tctggtccacagctacaacagg FM-TG- tctggtccacagctacaacaga	RW-*TG*- gatgtagtaggcaccctagccg
	Exon 7	
c.886 C >T	FW-TG- caatcagtcatctctggcagattcc FM-TG- caatcagtcatctctggcagattct	RW-TG- ggcggcagcttggaacaa
	Exon 34/Intron	
IVS34+1 G>C	FW-TG- ccttcggatggtaccagaagcccag FM-TG- ccttcggatggtaccagaagcccac	RW-TG- atcatggcacactgaagaagttg

Molecular analysis of *TG* mutations

Targeted DNA was amplified by three-LNA primer – PCR reaction using a modified primers one for wild type (normal primer) and the other complimentary to the mutation to be detected (mutation primer) (13,14). The presence of product in wild type and mutant was determine as heterozygousity, while the presence of only mutant band indicate the homozygousity of the mutation, while the presence of the wild type primer band only refer to normal position (no mutation) (15). The optimum reaction conditions of PCR was listed in table (2). The LNA primer-PCR products and the ladder marker were resolved by 1% agarose gel electrophoresis at 100 volt for 45 min. The gel was stained with ethidium bromide solution (0.5µg/ml) and visualized on UV transilluminator and then photographed by using Gene Flash gel documentation system (13).

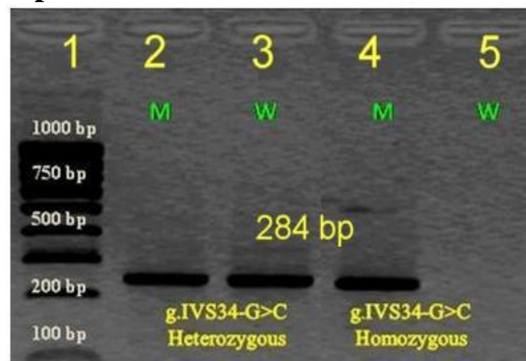
Table(2):The LNA PCR) cycles for amplification conditions

Program Step	Temperature	Time	No. of Cycles
Preheat	95 ºC	10 min.	1 cycle
Denaturation	95 ºC	30 sec.	30 cycles
Annealing	56 ºC	30 sec.	
Extension	72 ºC	30 sec.	
Termination	92 ºC	10 min.	1cycles
	30 ºC	3 min.	

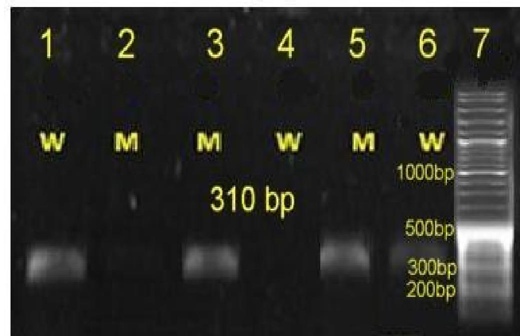
RESULTS AND DISCUSSION

Twenty six TG mutations were detected, 13(50%) in thyroid cancer and 13(50%) in toxic goiter. Among these mutations, 23 mutations were detected as heterozygous mutations(Data not seen) and 3 mutations as homozygous. Two of these homozygous mutations were detected in two toxic goiter patients as guanine to adenine transition g.IVS5+1G>A at position +1 of the donor splice acceptor site in exon-intron 5 figure (1) and transversion that replace guanine by cysteine (g.IVS34-1G>C) in the exon 34 figure (2). The third homozygous mutation was detected among one thyroid cancer patient as transition that replace cysteine by thymine c.886C>T in the exon 7 figure (3). Several thyroid disorders are associated with genetic defects of TG gene as well to other genes (16). Most of these defects were identified as mutations. Many of these mutations occurred in the absence of exons from the gene transcript because of transversion or transition at positions in the acceptor splice sites of many introns that replace the normal bases with abnormal ones (17,18). Such mutations were detected as a splice donor site mutation of intron 5 (g.IVS5+1G>A) or at position -1 in the acceptor site of intron 34(g.IVS34−1G>C) (4,5,19). The c.886C>T transition in exon 7 was also detected to produce a premature stop codon, which results in a truncated protein of 276 amino acids (R277X) with limited ability to generate thyroid hormone (20). Most of these mutations were identified as heterozygous with very rare homozygosity. In the present results, three homozygous mutations were detected, two of these were detected in two toxic goiter patients as g.IVS5+1G>A and g.IVS34-1G>C mutations. The third homozygous mutation was detected among one thyroid cancer patient as c.886C>T mutation. These homozygous mutations may reflect the severity and genetic instability of toxic goiter and thyroid cancer than other thyroid disorders especially that these mutations caused excision of exons which produced a severely truncated proteins or cause a hot spot mutation. Homozygous g.IVS5+1G>A, c.886C>T mutations, severity and genetic instability of toxic goiter and thyroid cancer were detected in a patients with recurrent goiter and a metastatic follicular thyroid carcinoma (4) and in goiter patients with congenital hypothyroidism (21). On the other hand, no homozygous g.IVS34-1G>C mutation was detected in thyroid disorders yet and we first time detect such homozygous mutation in one toxic goiter patient. Other TG homozygous mutations such as g.IVS30+1G>T, p.Q2142X, p.C1245R, p.C1977S and p.C1058R mutations were identified in patients with various thyroid disease or without thyroid disorder(6,7,8).

Figure(1):Ethidium bromide stained 1% agarose gel, showed TG gene homozygous g.IVS5+1G>A mutation among toxic goiter patients. Lanes:1,3,5 wild types(W) samples with no bands, Lanes: 2,4,6 mutant type(M) samples with 213 bp PCR product bands and Lane 9 marker.



Figure(2):Ethidium bromide stained 1% agarose gel, showed TG gene heterozygous and homozygous g.IVS34 G>C mutations among toxic goiter patients. Lane: 1 Marker, Lanes 2 and 4:mutant (M), Lanes 3 and 5:wild types (W) samples.



Figure(3):Ethidium bromide stained 1% agarose gel, showed TG gene heterozygous and homozygous c.886 C>T mutations among thyroid cancer patients. Lanes 1,4 and 6:wild types samples(W), Lanes 2, 3 and 5:cancer samples(M) (Lane 2 no mutation, Lane 3 homozygous mutation and Lane 5 heterozygous mutation) and Lane 7: marker.

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