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Detection of T.G. and TO Genes Compound Mutations Associated with Thyroid Carcinoma, Toxic Goiter and Hypothyroidism in Iraqi Patients

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Molecular genetic analysis of the T.G. and TO genes was carried out in many countries which resulted in the identification more than 35 effective mutations most of them clustered in the different region of T.G. and TO genes due to the high prevalence of the thyroid disorders. This study aimed to detect some compound mutations in T.G. and TO genes of Iraqi patients with thyroid disorders. PCR was used for detect eight common mutations that were chosen for diagnosis in this study using Locked Nucleic Acid (LNA) primers. In the current work results showed that 12 patients were with more than one mutation. Most of these mutations were detected in thyroid toxic goiter group 6(50%) followed by cancer group 5(41.7%) and one (8.3%) case in hypothyroidism. Furthermore, the frequency of the mutations compound TO c.1978C>G (40%) and (50%) T.G. g.IVS5+1 G>A with a second mutation were higher than other mutations. The results also showed that new compound heterozygous mutations were reported here in hypothyroidism, thyroid cancer and thyroid toxic goiter which include one compound heterozygous mutation c.986A>C and c.2610G>T, Six compound mutations g.IVS5+1G>A+1978C>G, one from each g.IVS5+1G>A/ g.IVS34-1G>C, c.986A>C/1978C>G, g.IVS5+1G>A/ c.886C>T, c.IVS10-1G>A/1978C>G, four compound mutations include g.IVS5+1G>A/ g.IVS34-1G>C, c.2610G>T /1708C>G, g.IVS34-1G>C /1708C>G and g.IVS5+1G>A+1978C>G, respectively. The increased incidence of compound mutations in thyroid toxic goiter and thyroid cancer patients indicate that the DNA instability is very high in those patients.

Key words: T.G., TO, compound mutations, thyroid disorders, thyroid cancer

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INTRODUCTION

Thyroid disorders are one of the most common endocrinal disorders. They arise after disturbances in thyroid hormonogenesis biosynthetic and regulation pathways (Cahmann *et al*., 1977). These biosynthesis pathways are rolled by several genes. Thyroglobulin (T.G.) and Thyroid peroxidase (TO) genes are the main keys in this biosynthesis. Initially T4 and T3 are separated from thyroglobulin encoded by T.G. gene then iodinated by thyroid peroxidase encoded by TO gene. This process is regulated by Thyroid Stimulating Hormone (TSH) released from the pituitary gland. Thyroid hormones disturbances are strongly associated with the thyroid disorders. Most of these disturbances are due to mutations in T.G. and TO genes. Previous reports were identified and characterized several mutations in the human T.G. and TO genes including missense mutations, splice site mutations, nonsense mutations and deletions (Targovnik *et al*., 1993; Targovnik *et al*., 2001; Hamosh *et al*., 2005; Park and Chatterjee, 2005; Alzahrani *et al*., 2006; Kanou *et al*., 2007; Yardena *et al*., 2007; Turkkahraman *et al*., 2010; http://www.ncbi.nlm.nih.gov/omim/188450). Compound heterozygous mutations were also detected in patients with thyroid disorders. Such mutations were identified in T.G. and TO genes in patients affected with affected various thyroid disorders (Targovnik *et al*., 1993; Van de Graaf *et al*., 1999; Bakker *et al*., 2000; Medeiros-Neto *et al*., 2002; Caron *et al*., 2003; Gutnisky *et al*., 2004; Carina *et al*., 2005; Mendive *et al*., 2005; Rivolta *et al*., 2005). We previously identified various T.G. and TO mutations associated with different types of thyroid disorders (Al-Faisal *et al*., 2012a; Al-Ramahi *et al*., 2012). In the present study, we extended our initial molecular studies to detect compound mutations of T.G. and TO genes associated with thyroid disorders.

MATERIALS AND METHODS

Subjects: One hundred and nine Arabic patients (31 males and 78 females) with thyroid disorders (29 Hypothyroidism, 17 Thyroid cancer, 31 Thyroid toxic goiter and 32 Thyroid nontoxic goiter) were enrolled in this study (They attended the endocrinologist in Nuclear Medicine Hospital and Al Yarmok Nuclear Medicine Department in Baghdad, Iraq). Clinical, ultrasonication and serum thyroid hormones were used for diagnosis. Twenty five (12 males and 13 females). Arabic healthy individuals who served as a control were selected. Healthy controls and patients' ages ranged from 17 to 79 years. The study was carried out in Baghdad, Iraq from July 2009 to January 2012.

Inclusion and exclusion criteria: The inclusion and exclusion criteria were applied as follows:

- **Inclusion criteria:** Patients of any age or sex with known or suspected thyroid abnormalities and newly diagnosed patients
- **Exclusion criteria:** Children, pregnant or lactating women, treated patients, patient with cardiac problems or neurological disorders

Blood samples: Five milliliter of venous blood sample was collected by trained nurses from each individual of both thyroid patients and normal. All samples were obtained after informed consent of the participants prior to their inclusion in the study. A structured questionnaire was used to elicit detailed information on age, affected side, residence, type of feeding and family history of thyroid disorders.

Ethical use of data: Informed consent was obtained from all the study participants and the guidelines set by the ethics committee of our institute and hospitals were applied.

Isolation of genomic DNA: Three milliliters venous blood was collected in EDTA tubes and genomic DNA was extracted according to DNA extraction kit protocol from Promega, USA. The DNA concentration and purity were estimated according to Sambrook *et al*. (1989).

T.G. and TO genes mutations: Eight common mutations were chosen for diagnosis in this study. The selection of these mutations is based on the distribution of the most common mutations associated with thyroid disorders. These mutations include g.IVS5+G>A, c.886C>T, c.986A>C, c.2610G>T, g.IVS10-1G>A and g.IVS34-1G>C located in exons 5, 7, 8, 10 and 34 of T.G. gene, respectively (Corral *et al*., 1993; Gutnisky *et al*., 2004; Hishinuma *et al*., 2005; Rivolta *et al*., 2005; Alzahrani *et al*., 2006). While 1708C>T and 1978C>G located in exons 10 and 11, of TO gene, respectively (Bikker *et al*., 1997; Kotani *et al*., 1999).

Locked nucleic acid primer PCR: Locked nucleic acid (LNA) primer PCR was carried out according to Obika *et al*. (1998), Singh *et al*. (1998) and Koshkin *et al*. (1998). The mutations primers were designed and identified using NCBI tools (Table 1). The design of these primer sequences was done using GeneFisher and OligoAnalyzer (https://www.idtdna.com/analyzer/Applications/OligoAnalyzer; http://lnatools.com) and primer BLAST programs.

Table 1: Sequences for primer sets used for PCR amplification of human T.G. and TO genes to detect some mutations

Gene	Exon	Mutation	Forward LNA primer	Reverse LNA primer
T.G.	Exon 5/Intron	g.IVS5+1G<A	FW-T.G.-5 tctggtccacagctacaacagg	RW-*T.G.*-7 gatgtagtaggcaccctagccg
			FM-T.G.-6 tctggtccacagctacaacaga	
	Exons 7 & 8	c.886 C >T	FW-T.G.-8 caatcagtcatctctggcagattcc	RW-T.G.-10 ggcggcagcttggaacaa
		c.986A>C	FM-T.G.-9 caatcagtcatctctggcagattct	RM-T.G.-11 ggcggcagcttggaacac
	Exon 10/Intron	c.2610G>T	FW-T.G.-1c gaagggaaacggccccag	RW-T.G.-3b ctcttcatatgcgttagctcag
		g.IVS10+-1 G>A	FM-T.G.-2b gaagggaaacggccccat	RM-T.G.-4b ctcttcatatgcgttagctcaa
	Exon 34/Intron	IVS34+1G>C	FW-T.G.-12 ccttcggatggtaccagaagcccag	RW-T.G.-14 atcatggcacactgaagaagttg
			FM-T.G.-13 ccttcggatggtaccagaagcccac	
TO	Exon 10	1708 C> T	FW-TO-21 gtggtttggacccactaatac	RW-TO-23 cctgggaggttcagaacc
			FM-TO-22 gtggtttggacccactaatat	
	Exon 11	1978 C>G	FW-TO-24 cctggacttgtacaagcatcc	RW-TO-26 cgtcccattctaagtgctacg
			FM-TO-25 cctggacttgtacaagcatcg	

W: Wild type, M: Mutation, LNA: Locked nucleic acid

Table 2: PCR conditions used for PCR amplification of *T.G.* and *TO* genes

Program step	Temperature	Time	No. of cycles
Preheat	95 °C	10 min	1 cycle
Denaturation	95 °C	30 sec	
Annealing	56 °C	30 sec	
Extension	72 °C	30 sec	30 cycles
Termination	92 °C	10 min	
	30 °C	3 min	1cycles

Molecular analysis of T.G. and TO mutations: Targeted DNA was amplified by two LNA primer PCR reactions using modified primers: one for wild type (normal primer) and the other complimentary to the mutations to be detected (mutation primer) (Old, 1996; Ye *et al*., 2001). The presence of product in wild type and mutant was determined as heterozygosity, the presence of only mutant band refers to homozygosity of the mutation and the presence of the wild-type primer band only refers to normal position (no mutation) (Najmabadi *et al*., 2001).

The optimum reaction conditions of PCR were listed in Table 2. The LNA primer PCR products and the ladder marker were resolved by 1% agarose gel electrophoresis at 100 V for 45 min. The gel was stained with an ethidium bromide solution (0.5 μg mL^{-1}) and visualized on a UV transilluminator then photographed.

RESULTS AND DISCUSSION

T.G. and TO genes are the most important thyroid genes that are associated with thyroglobulin iodination process for thyroid hormones synthesis. The failure of this process led to a variant type of disease such as adenomatous goiter, congenital hypothyroidism, simple goiter, multi toxic or nontoxic nodules and cold or hot thyroid nodules (Baczyk *et al*., 2009). Researchers from many countries far away from Iraq such as Japan, Taiwan, Argentina, Spain, Germany (Corral *et al*., 1993; Hishinuma *et al*., 1999; Hishinuma *et al*., 2005) and near closely like Turkey, Israeil and Saudia Arabia (Alzahrani *et al*., 2006; Yardena *et al*., 2007) identified a bout thirty five mutations in T.G. gene and fifty in TO gene (Hamosh *et al*., 2005) led to thyroid disorders in human.

Table 3: Compound mutations detected in patients with thyroid disorders

Groups	Patients No.	Types of mutations
Hypothyroidism	1	c.986A>C+c.2610G>T
Thyroid cancer	2	g.IVS5+1G>A+ g.IVS34-1G>C
	1	c.2610G>T+1708C>T
	1	g.IVS34-1G>C+1708C>T
	1	g.IVS5+1G>A+1978C>G
Thyroid toxic goiter	1	g.IVS5+1G>A+ g.IVS34-1G>C
	1	c.986A>C+1978C>G
	1	g.IVS5+1G>A+c.886C>T
	1	g.IVS10-1G>A +1978C>G
	2	g.IVS5+1G>A+1978C>G
Total	12	

The current work results were illustrated in Table 3 (Fig. 1, 2). The results showed that 12 patients were with more than one mutation. Most of these mutations were detected in thyroid toxic goiter group 6(50%) followed by cancer group 5(41.7%) and one (8.3%) case in hypothyroidism. Furthermore, the frequency of the mutations compound TO c.1978C>G (40%) and (50%) T.G. g.IVS5+1 G>A with a second mutation were higher than other mutations.

The results also showed that new compound heterozygous mutations were reported here in hypothyroidism, thyroid cancer and thyroid toxic goiter which include one compound heterozygous mutation c.986A>C and c.2610G>T, Six compound mutations g.IVS5+1G>A+1978C>G, one from each g.IVS5+1G>A/ g.IVS34-1G>C, c.986A>C/1978C>G, g.IVS5+1G>A/ c.886C>T, c.IVS10-1G>A/1978C>G, four compound mutation include g.IVS5+1G>A/g.IVS34-1G>C, c.2610G>T /1708C>G, g.IVS34-1G>C /1708C>G and g.IVS5+1G>A+1978C>G, respectively.

The compound mutations detected in this work revealed that the DNA instability is very high in thyroid toxic goiter and thyroid cancer. More evidence about DNA instability in thyroid toxic goiter and thyroid cancer were also detected in this study (Data not published yet). Two homozygous mutations were detected in toxic goiter and one homozygous mutation in thyroid cancer, which reflect the possible loss of heterozygosity. Other evidence come from the total of mutations detected among the thyroid groups where the highest number of mutations

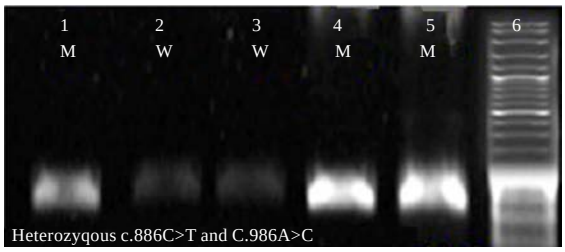

Fig. 1: Ethedium bromide stained 1% agarose gel, screening of DNA thyroid disorders samples for T.G. gene heterozygous of c.886c>T and c.986>C mutations by LNA-primer PCR for 45 min at 100 volts. The wild type bands are present in all PCR products (310 bp) for mutant primer and the sequence of wild and mutant types. Line 1: Thyroid carcinoma sample, Lines 4 and 5: Toxic goiter samples, Line 6: Lader

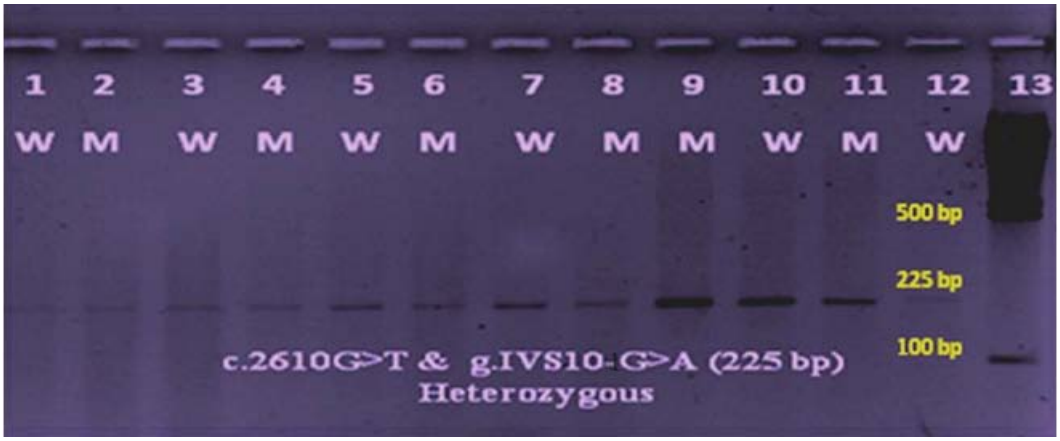

Fig. 2: Ethedium bromide stained 1% agarose gel, screening of DNA thyroid disorders samples for T.G. gene heterozygous c.2610G>T and g.IVS10-1G>A mutations by LNA-primer PCR for 45 min at 100 volts. The wild type bands are present in all PCR products (225 bp) for mutant primer and the sequence of wild and mutant types. Lines 2 and 4: Thyroid carcinoma samples, Lines 6 and 8: Toxic goiter samples, line 9 and 11: Hypothyroidizm samples, Line 13: Lader

were recorded in thyroid toxic goiter (27 mutations) and thyroid cancer (21 mutations) (Data not published yet). The evidence indicates that thyroid toxic goiter could be the final step to produce thyroid carcinoma. The high DNA instability associated with thyroid disorders which was detected in this study is in parallel to our previous results (Al-Faisal *et al*., 2012a, b; Al-Ramahi *et al*., 2012) which may reflect the possibility of chronic exposure to genotoxic agents such as radiations. After Gulf Wars the Iraqi environment radiation contamination was well documented as well to the incidences of various types of cancer (Al-Azzawi and Al-Saji 1998; Al-Azzawi *et al*., 1999, 2002; Ali and Al-Ali 2002; Al-Sadoon *et al*., 1998; Butrus *et al*., 2002; IFAM, 1995; Yaqoub *et al*., 1998a, b, 1999, 2002). The DNA instability in thyroid disorders patients associated with radiation was also detected by others (Livingston *et al*. 1993; Gutierrez *et al*., 1997, 1999; Sbrana *et al*., 2006; Brooks *et al*., 2007; Dardano *et al*., 2007; Tronko *et al*., 2007; Hooman *et al*., 2008; Scarpato *et al*., 2009; Cardis and Hatch, 2011; Medalia, 2011).

Compound mutations in T.G. and TO genes were also detected by others. Caron *et al*. (2003) who reported that the compound heterozygous mutation in the T.G. gene was identified in a family with affected siblings with congenital goitrous hypothyroidism. Pervious molecular analyses revealed that p.R277X/p.R1511X compound heterozygous mutations have been found before causing T.g. deficiency (Targovnik *et al*., 1993; Van de Graaf *et al*., 1999; Gutnisky *et al*., 2004; Mendive *et al*., 2005; Rivolta *et al*., 2005). On the other hand Medeiros-Neto *et al*. (2002) indicated that the affected individuals are either compound heterozygous for R277X/IVS34–1G>C or R277X/R1511X.

DNA instability, loss of heterozygosity and more recently, gene arrangement and chromosomal translocations are considered as an important step in

carcinogenesis and have investigated as potential markers to discern benign from malignant disease (Krohn and Paschke, 2001).

Thyroid cancer, the most common endocrine cancer, has been shown to display a high genetic instability (Kim *et al*., 2005). More than 50 mutations among T.G. and TO genes were identified and correlated with different types of thyroid disorders (http://www.ncbi.nlm.nih.gov/omim/188450). Another genetic alteration in the thyroid cancer is LOH. LOH represents the normal function of one allele of a gene in which the other allele was already inactivated (Rodrigues-Serpa *et al*., 2003). LOH is detected on average in 30-50% of follicular thyroid carcinoma (Doboz *et al*., 2000) and the frequency of LOH has been found to be correlated with the aggressiveness of the tumor and the presence of relapse in patients with thyroid cancer (Targovnik *et al*., 1993; Targovnik *et al*., 2001; Kanou *et al*., 2007).

DNA instability was also identified in goiter. Toxic goiter has been found to increase the risk to develop a thyroid lympho proliferative disease such as thyroid lymphoma (Giusti *et al*., 2010) or hyperplasia (Gimm, 2001).

CONCLUSION

Twelve patients have more than one mutation (compound), 11 of them were from patients with thyroid toxic goiter and thyroid cancer and these results indicate that the DNA instability is very high in thyroid toxic goiter and thyroid cancer patients.

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