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Evaluation of Forkhead Box Protein E1 *(FOXE1)* Gene Expression and Thyroid-Stimulating Hormone (TSH) Levels in a Sample of Hyperthyroidism Iraqi Patients

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Abstract: Hyperthyroidism refers to a type of common endocrinology disease in which the level of thyroid hormone in the body is abnormally increased. Although several clinical studies have researched the genetic factors of hyperthyroidism the influence of thyroid hormones on gene expression are still unclear. Forkhead box (FOX) family proteins regulate transcription and DNA repair and are involved in cell growth, differentiation, embryogenesis, and lifespan. The transcription factor *FOXE1* is a member of the FOX family. The relationship between the expression level of *FOXE1* and Hyperthyroidism prognosis remains controversial. This study aimed to estimate the levels of *FOXE1* gene expression in a sample of Iraqi patients with Hyperthyroidism**.** In a case-control study included 48 Iraqi patients suffered from hyperthyroidism**,** before and after a single dose of oral sodium iodide I^{131}- capsule treatment and 50 **s**eemingly healthy volunteers as a control group with age range from (15-75) years were enrolled in this study during their attendance at the Alamal Alwataniy Hospital in Baghdad from the period July 2022 to the middle of March 2023. Blood samples were collected to evaluate the level of thyroid stimulating hormone (TSH) automatically by Cobas e411 (Hitachi/Roche) device and RNA was extracted from whole blood samples, followed by cDNA synthesis. Subsequently, the levels of *TSHR* transcripts were measured by using Real time Polymerase Chain Reaction (RT-PCR). The *FOXE1* expression level, exhibited a substantial upregulation in the whole blood samples obtained from patients after treatment 2.63±0.61) than before treatment 2.10 ±0.52) compared to those obtained from healthy controls. The current study concluded that *FOXE1* may be a potential independent prognostic factor for Hyperthyroidism patients.

Keywords: Hyperthyroidism, *FOXE1*, gene expression, Real-time PCR

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Introduction

Hyperthyroidism is classified as an autoimmune disorder that primarily affects the thyroid gland. This clinical condition is characterized by the hyperproduction and excessive secretion of thyroid hormones by the gland(1). The thyroid gland necessitates specific physiological conditions for the biosynthesis of its hormones, which are influenced by a variety of regulatory factors. Disruptions in these regulatory mechanisms can lead to a spectrum of thyroid disorders. Thyroid hormones play a crucial role in metabolic processes and overall developmental pathways. Dysregulation of thyroid function may manifest as either hypersecretion or hyposecretion of these hormones(2). Sufficient levels of thyroid hormones (TH) are crucial for normal growth, cellular differentiation, regulation of energy metabolism, and the physiological integrity of nearly all

human tissues. The significance of these hormones is exemplified by the marked clinical manifestations observed in both hypothyroidism and hyperthyroidism (3). Hyperthyroidism, a condition often classified as an autoimmune disorder, is characterized by the pathological overproduction and secretion of thyroid hormones from the thyroid gland (1). This condition exhibits a global prevalence estimated between 0.2% and 1.3% (4). Genetic predispositions, particularly the regulation of specific genetic components, play a substantial role in the pathogenesis of thyroid diseases (5). A prominent candidate gene demonstrating low to moderate penetrance is Forkhead box E1 (*FOXE1*), which belongs to a broader family of transcription factors. Previously referred to as thyroid transcription factor 2 (*TTF2*), *FOXE1* is recognized as a fundamental protein vital for thyroid gland functionality. It is expressed during the embryonic development of the thyroid primordial and continues to be expressed throughout the gland's development. *FOXE1* is implicated in numerous processes involving thyroid follicular cells. The thyroid-specific genes regulated by *FOXE1* are critical for both the development of the thyroid gland and the migration and differentiation of thyroid cells (6). This intronless gene is located on the long (q) arm of chromosome 9 at position 22 and is characterized by a distinctive forkhead domain. As a thyroid transcription factor, *FOXE1* is likely essential for thyroid morphogenesis. Considering the significant health ramifications associated with hyperthyroidism, the present study aims to assess the expression of the *FOXE1* gene in a cohort of Iraqi patients diagnosed with hyperthyroidism.

Material and methods

Study design

This study is a case–control study that consisted of 98 individuals from Iraq with ages ranging between (15-75 years) during the period between July 2022 and the middle of March 2023, categorized into two distinct groups: patients and controls. The patient group consisted of 48 individuals (21 males and 27 females) suffering from hyperthyroidism after being diagnosed by the physician and who were taking treatment for the first time and were successfully followed up before and after a single I^{131} dose oral capsule treatment as a routine procedure. These participants were selected from Alamal Alwataniy Hospital in Baghdad city. Also, the study included 50 (17 males and 33 females) apparently healthy volunteers as a control group, obtained from the National Blood Transfusion Centre, who had never had thyroid disorder or other chronic diseases.

Sample collection and laboratory investigations

From everyone participating enrolled in this study, 4 ml of blood was drawn by venipuncture using disposable syringes. Each sample was divided into two parts, two ml of blood were used for genetic analysis transferred into an EDTA anticoagulant tube with gentle mixing, a portion of 500 µl was immediately added to a 1.5 ml tube containing 700 µl of TRIzol; the tube was inverted many times for mixing and kept at -20 °C until it was subjected to RNA extraction. *FOXE1 gene* expression was estimated by using real-time PCR (RT-PCR) and Three ml of blood was put into a sterile gel vacuum tube and left to stand for 10–20 minutes at room temperature (25°C), then centrifuged at 3000 rpm for 5 minutes, to get serum for TSH hormonal analysis

by using a fully automated analyzer Cobas e411 (Hitachi/Roche).

Evaluating the *FOXE1* gene's expression

RNA was extracted from blood samples according to the protocol of TRIzol™ Reagent. Amount 500 µl of blood were obtained from each subject and put into 750 µl of Trizol preservation Reagent (Solar Bio, China) for RNA extraction, the cells in the sample were lysed by vortexing multiple times, followed by 10-minute incubation at room temperature. To extract the RNA containing aqueous phase, 0.15 mL of chloroform was added. Subsequently, 0.45 mL of isopropanol was introduced to precipitate the RNA, resulting in a white gel-like pellet. The washing of the RNA was carried out by adding 0.75 mL of 75% ethanol. Lastly, the pellet was rehydrated in 20 ul of RNase- Free water and incubated at 60C for 15 minutes by using thermomixer. Total RNA samples stored at -20 ºC until processed to downstream application. The purity and concentration of the extracted RNA were measured using a Qubit 4.0 (Invitrogen, USA). Expression of *FOXE1* gene was estimated by using a two-step RT-qPCR approach. In the first step, RNA was converted to cDNA utilizing the Add Script cDNA synthesis kit (NEB®, USA) according to program are shown in (Table1). Subsequently, in the second step, the RT-qPCR was carried out following the method outlined by (7), employing the specific primers provided by Macrogen (Korea) as depicted in (Table2), relying on information from NCBI. The PCR amplification was done by using RT-qPCR (Molecular System/Australia) according to program which clarified in (Table3). The expression of the *B2M* was employed as an endogenous control for the purpose of normalizing the data. The fold change in *TSHR* expression between the patient and the healthy group was determined by calculating the comparative relative quantification (RQ) level using the $2^{-\Delta\Delta Ct}$ method.

Table (1): Thermal cycler steps for cDNA reverse transcription conditions

Steps	Temperature (°C)	Duration	Cycles
Priming	25	10 min	1
Reverse transcription	42	60 min	
RT inactivation	80	5 min	
hold	4 ∞	-------	

Table (2): Primer Sequence for *FOXE1 and their housekeeping B2M* Gene Expression

Primer Name	Sequence (5′→3′ direction)	Tm °C	Repeat cycle
FOXE1	GATGCTGCCCTGCGTATTTG	60	150
FOXE1	TAATTGCTGCCTGGAGCCAA		
B2M-F	CTGGGTTTCATCCATCCGACA	60	139
B2M--R	TCAGTGGGGGTGAATTCAGTG		

Table (3): The thermal profile of *FOXE1 and B2M* gene expressions

Steps	Temperature (°C)	Duration	Repeat cycle
Initial Denaturation	95	60 sec	1
Denaturation	95	15 sec	40- 45
Annealing	60	30 sec	
Extension	60-95	40 min	1

Ethical approval

This study was approved by the Council of the Institute of Genetic Engineering and Biotechnology / University of Baghdad, and the study protocol was approved by the Ethics Committee of the Iraqi Ministry of Health and Environment according to the document number H T 24919 (including the number and the date in 20/6/ 2022) to get this approval.

Statistical analysis

It was done using International Business Machine Statistical package for the Social Sciences (IBM SPSS–version 28) was used to detect the effect of difference factors in study parameters. Least significant difference –LSD test (Analysis of Variation-ANOVA) was used to compare significance compare between means. Statistical significance was considered whenever the P value was equal or less than 0.05 and highly significant whenever the P value was equal or less than 0.01.

Results

Age Distribution of the Studied Groups

The study consisted of participants spanning various age groups, ranging from 15 to 75 years old. To generate more accurate and efficient results, the age of the studied groups was categorized into five homogeneous age groups: (15–45), (46-75). The results of distributing age groups according to disease status (patients Vs controls) showed that 47.92% of the patients and 70% of the controls were in the 15-45 years' age group while 52.08 % of the patients and 30 % of the controls were in the 46-75 years' age group, these values differed statistically significantly (p=0.026) as shown in table (4).

Table (4): Age distribution in hyperthyroid patients and control subjects.

Age groups	Patients No. 48 (%)	Control No. 50 (%)
15-45 (years)	23 (47.92)	35 (70.0)
46-75 (years)	25 (52.08)	15 (30.0)
Chi square (x 2)	4.944	
P-value	0.026*	
*SignificantatP≤0.05		

Level of thyroid stimulation hormone

Table (5) summarizes the results of the TSH levels in the patients with hyperthyroidism before and after treatment. There was a highly significant ($p \leq 0.01$) increase in the level of TSH in patients with hyperthyroidism after treatment group (14.553 ± 5.08 μIU/ml) than before treatment group (0.087 ± 0.04 μIU/ml).

Table (5): thyroid stimulation hormones level in hyperthyroid patients

Group				
Hormone level	**Before TR**	**After TR**	**T-test**	**P-value**
TSH (μIU/ml)	0.087 ± 0.04	14.553 ± 5.08	-2.842	0.009**
(P≤0.01)HighlySignificant**				

***FOXE1* gene expression**

In the present study, the result of the comparison of *FOXE1* gene expression in patients (before and after treatment) and control groups revealed a substantial slightly increase in *FOXE1* average folding in patients after treatment than in patients before treatment compared to the control group (2.63), (2.10), and (5.06), respectively. (Table 4) summaries the expression level of *FOXE1 gene* mRNA in patients (before and after treatment) and controls by the $2^{-\Delta\Delta Ct}$ method.

Table (6): Fold of *FOXE1* expression depending on 2-ΔΔCt method

Group	Means Ct of *FOXE1*	Means Ct of *B2M*	ΔCt	ΔΔCt	Fold of gene expression
Patients before treatment	23.13±0.37	16.33±0.36	6.80±0.39	7.05±0.39	2.10^{a}±0.53
Patients after treatment	24.12±0.54	16.95±0.46	7.16±0.36	0.45±0.36	2.63^{a}±0.61
Control	23.55±0.86	17.03±0.64	6.52±0.59	0.0002±0.59	5.06^{b}±1.13

Discussion

The observed pattern corresponds with numerous significant studies examining age-associated trends in thyroid dysfunction and also highlights certain population-specific traits. The increased prevalence of hyperthyroidism in older age groups (46-75 years) is inconsistent with findings from a 2021 study that identified maximum prevalence in the 40-49 years' age range (8). Additionally, aged hyperthyroid individuals (≥70 years) generally have less classical signs but demonstrate increased incidences of weight loss, atrial fibrillation, and apathy instead of hyperactivity (9). Before that, (10) divided the papillary thyroid cancer patients into two subgroups with the reference to the age at diagnosis: ≤ 45 years, and > 45 years.

Radioactive iodine (RAI) treatment is a prevalent intervention for hyperthyroidism, especially in instances of Graves' illness. The procedure entails the administration of radioactive iodine-131 to obliterate a portion or the entirety of the thyroid gland, thereby diminishing hormone synthesis. The influence of RAI on blood TSH levels can differ markedly pre- and post-treatment (11). In hyperthyroid patients, the levels of TSH are often decreased due to feedback inhibition from increased thyroid hormones (T3 and T4). This suppression results in diminished or negligible TSH levels (12). Following therapy, the diminished activity of the thyroid gland often leads many individuals to acquire hypothyroidism, necessitating permanent levothyroxine medication. This alteration leads to increased TSH levels as the body endeavors to activate the rest of the thyroid tissue (13). Besides, during the first twelve months following radioactive iodine treatment, particularly in the initial six months, a team of researchers found that patients frequently experienced aberrant thyroid hormone levels (14). In line with a local study finding concerning the measurement of TSH serum concentrations, a highly significant difference was found between controls and those with hyperthyroidism (15). Additionally, an unusual TSH response was noted in another local study examining the effects of low doses of radiation on thyroid gland activity (16).

The results of the study suggest that *FOXE1* gene expression in hyperthyroid patients, both pre- and post-treatment, lacks significant changes when compared to one another; yet, both patient groups show significant differences from the control group. It implies that *FOXE1* expression may be impacted by the hyperthyroid state rather than the effects of treatment. The findings of this study coincide with prior work demonstrating that *FOXE1* is modulated by thyrotropin (TSH) and transforming growth factor-beta (TGF-β), which are vital to the expression of thyroid differentiating genes. This suggests that hormonal regulation

greatly influences *FOXE1* expression, potentially addressing why therapy did not considerably modify its levels (17). Recent investigations indicate that *FOXE1* is essential for thyroid development and cancer histology. Decreased *FOXE1* dose results in less aggressive thyroid tumors characterized by reduced proliferation and enhanced apoptosis, highlighting its influence on tumor differentiation (18). While research on alterations in *FOXE1* gene expression during hyperthyroidism treatment is scarce, overall findings indicate that therapies such as radioactive iodine or antithyroid medications normally concentrate on lowering thyroid hormone synthesis rather than directly modifying transcription factors (19).

Conclusion

This study's findings indicate a significant upregulation of *FOXE1* transcript levels observed in the whole blood samples obtained from patients diagnosed with Hyperthyroidism, compared to the control group consisting of healthy individuals.

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