STUDY THE EXPRESSION OF IL-22 GENE IN AUTOIMMUNE HYPOTHYROIDISM IN IRAQ

A. H.Yassin[1,4] A. A. Al-Kazaz[1] A. M. Rahmah[2] T. Y. Ibrahim[3]
Researcher Prof. Prof. Researcher
[1]Biotect. Dept., Coll. of Sci., Univ. of Baghdad [2]National Diab. Cent., Univ. of Mustansiriyyah.
[3]Ministry of Industry, Ibn Sina Rese. Cent. [4]Medical Lab. Tech. Dept., Dijlah Univ. Coll.
E-mail: alyaa.hamza@duc.edu.iq

ABSTRACT

This study was aimed to assess expression of IL-22 gene in patients with newly diagnosed autoantibodies against the thyroid antigens; anti-thyroglobulin (anti-TG), and anti-thyroid peroxidase (anti-TPO) in comparison with patients previously diagnosed and treated for autoimmune hypothyroidism as well as healthy control individuals. Twenty-eight patients with positive anti-TG and/or anti-TPO were enrolled in this study along with twelve age- and gender-matched healthy controls. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was used to assess gene expression and fold change among the study groups. Results revealed anti-TG concentration ranges of 134.3 to 2998.9 IU/mL and anti-TPO concentration ranges of 41.6 to 1018.4 IU/mL in patients while control group individuals were negative for these autoantibodies. The mean fold change of IL-22 expression ($2^{-\Delta\Delta Ct}$) was the highest in newly diagnosed patients compared to patients on treatment for autoimmune hypothyroidism and healthy controls indicating the potential role of IL-22 in the early stages of the disease and its contribution in immune imbalance.

Keywords: Gene expression, interleukin 22, thyroid gland, qRT-PCR.

دراسة التعبير للمورث بين ابيضاض 22 في مرض قصور الغدة الدرقية المناعي الذاتي في العراق

علياء حمزة ياسين[4،1] عبد الكريم عبد الرزاق القزاز[1] عباس مهدي رحمة[2] طه ياسين ابراهيم[3]
باحث استاذ استاذ باحث
[1]قسم التقنيات الاحيائية / كليةالعلوم / جامعة بغداد [2]المركز الوطني لبحوث وعلاج السكري / الجامعة المستنصرية
[3]مركز ابحاث ابن سينا / هيأة البحث و التطوير الصناعي / وزارة الصناعة [4]قسم تقنيات المختبرات الطبية / كلية دجلة الجامعة

المستخلص

تم إجراءهذه الدراسة لتقييم مستوى التعبير لمورث البين ابيضاض 22 في عينة من مرضى قصور الغدة الدرقية المناعي الذاتي في العراق و المشخصين حديثا بتكوين الاجسام المضادة الذاتية الموجهة ضد مستضدات الغدة الدرقية Anti-TG و Anti-TPO بالمقارنة مع مستوى التعبير لمجموعة من المرضى المشخصين مسبقا بالمرض و ملتزمين بالعلاج و مجموعة من الافراد الاصحاء كمجموعة سيطرة. شملت الدراسة ثمان وعشرون مريض واثنا عشر فردا كمجموعة سيطرة و تم تحديد مستوى التعبير للمورث بطريقة النسخ العكسي في تفاعل البلمرة المتسلسل الكمي (RT-qPCR) لقياس مضاعفات التعبير بين المجاميع. اظهرت النتائج وجود ارتفاع ذو فرق معنوي بين المجموعتين الاولى و الثانية مع مجموعة السيطرة من حيث نسبة تركيز الاجسام المضادة و التي تراوحت بين 134.3 الى 2998.9 IU/ml بالنسبة للجسم المضاد anti-TG و 41.6 الى 1018.4 IU/ml بالنسبة للجسم المضاد anti-TPO بينما اظهر افراد السيطرة نتيجة سالبة لهذه الاجسام المضادة، اما من ناحية مستوى التعبير لمورث البين ابيضاض 22 فقد كانت مجموعة المرضى الاولى ذات اعلى متوسط تعبير مقارنة مع مجموعة المرضى المشخصين مسبقا ومجموعة السيطرة، مما يدل بان البين ابيضاض 22 قد يكون له دور في المراحل المبكرة من المرض وقد يساهم في اختلال التوازن المناعي للجسم.

الكلمات المفتاحية: الغدة الدرقية، الاجسام المضادة الذاتية، التعبير الجيني، جهاز البلمرة

Received:13/8/2021, Accepted:21/11/2021

INTRODUCTION

The thyroid gland is a member of the endocrine system located in the front of the neck and responsible for the production and secretion of two metabolism-regulating hormones: triiodothyronine (T3) and thyroxine (T4) (2). Autoimmune thyroid disease (AITD) is a term for chronic autoimmune thyroiditis resulting from autoreactive lymphocytes infiltrating the thyroid gland. Among these is Hashimoto's thyroiditis (HT) which can be described as the destruction of thyrocytes by the autoimmune response which eventually develops into hypothyroidism. HT is named after Dr. Hakaru Hashimoto who diagnosed it for the first time in 1912 (15). The hallmark of AITD is the presence of autoantibodies against thyroglobulin (TG) and thyroid peroxidase (TPO) antigenic components of the thyroid gland. These autoantibodies have a diagnostic value for thyroid diseases, e.g. postpartum thyroiditis, Hashimoto's thyroiditis and Graves' Disease (21). Both genetic and environmental factors contribute to the etiology of AITD even though the specific causative pathogenesis and interaction between these factors are not clearly known. Susceptibility genes for AITD can be classified as either immune-modulating genes or thyroid specific (12). Dysfunction of T lymphocytes subsets or abnormal expressions of their cytokines may lead to the immune tolerance breakdown and imbalance resulting in abnormal immune reactions during AITDs development (13). There is no clear evidence of the exact DNA transcription and translation mechanisms as they are influenced by many factors such as transposons, ribosomes, inhibiting factors and epigenomics (6). CD8+ cytotoxic T lymphocytes are the leading cause of thyrocyte destruction in Hashimoto’s thyroiditis by antibody-mediated immune processes, with help from differentiated CD4+T helper cells (Th) (17). CD4+ T helper lymphocytes subsets have a key role in many autoimmune diseases pathogenesis. Among these is Th17 cells, producing interleukins 17A, 17F, 21and 22, which have been found in enhanced levels in Hashimoto's thyroiditis (7). Another CD4+ is Th22 which is the main expression and secretion source of IL-22 (18). In addition to their vital role in defense against microorganisms, Th22 cell and its effective factor IL-22 have been found to be implicated in the pathology of several autoimmune inflammatory diseases like multiple sclerosis, rheumatoid arthritis, immune thrombocytopenia, ankylosing spondylitis as well as thyroid autoimmunity (19) IL-22 belongs to IL-10- family of, mucosal epithelial cell proteins and acts via a transmembrane heterodimeric receptor complex of two subunits: IL-22R1 and IL-10R2 (4). In HT patients, an elevated number of cells Th17, with higher levels of IL-17 mRNA in their peripheral leucocytes along with a higher immunohistochemical IL-17 and IL-22 expression of in tissue of the thyroid have been adressed (5). Blood samples can be used as specimens for measuring gene expression levels of genes expressed by the immune cells to evaluate differences in expression levels between patients and healthy controls and to study the role of these genes in the development of diseases (1). This study was aimed to measure the level of IL-22 expression in the early stages of HT in comparison to autoimmune hypothyroid patients on thyroxine treatment and healthy controls.

MATERIALS AND METHODS

Subjects: In this study, 28 patients (3 males and 25 females) with positive anti-TG and/or anti-TPO were enrolled along with 11 (2 male and 9 females) age- and gender-matched anti-TG and anti-TPO negative healthy controls, all with an age range of 20 - 63 years. According to the status of thyroxine replacement therapy and autoimmune hypothyroidism development, patients were classified into two groups; group A: patients newly diagnosed with autoimmune response against the thyroid gland, and not receiving levothyroxine (n = 13, 2 male and 11 females) and group B: patients previously diagnosed with autoimmune hypothyroidism and receiving levothyroxine (n = 15, 1 male and 14 females). Autoimmune hypothyroidism diagnosis and levothyroxine doses were established by the consultant endocrinologist medical staff of the National Diabetes Center (NDC) located at Baghdad / Yarmouk, where all patients included in this study were attending for routine medical care provided by this center. Exclusion criteria included any history of malignancies and

treatment for hyperthyroidism, thyroid cancer or treatment with radioactive iodine. Informed consent was obtained in all subjects and the study was approved by the ethics committee in College of Science - University of Baghdad.

Thyroid function: All subjects underwent evaluation of thyroid function by measurement of serum thyroid-stimulating hormone (TSH), Triiodothyronine (T3) and Thyroxine (T4) using VIDAS kits (Biomerieux, France). Normal serum ranges were as follows: 0.35–4.94 lU/mL for TSH, 0.92–2.33 nmol/L for T3 and 60–120 nmol/L for T4. On the other hand, serum levels of anti-thyroid peroxidase (anti-TPO) and anti-thyroglobulin (anti-TG) autoantibodies were measured by enzyme-linked immunosorbent assay (ELISA) technique using Anti-TG and Anti-TPO kits (Aeskulisa, Germany). Negative results were considered if anti-TPO titer was <40 IU/mL and anti-TG titer was <120 IU/mL. All procedures were applied according to the manufactures instructions.

Blood sampling: Five to ten milliliters of venous blood from all patients and the control group were collected by vein puncture using a 10 mL syringe. A volume of 400 µL was transferred into Eppendorf tubes containing 600 µL of TRIZOL and was stored frozen at -20°C until RNA extraction. The remaining blood was placed in gel tubes and centrifuged for the separation of serum which was stored frozen (-20˚C) until use.

Isolation of RNA: isolation of total RNA from blood samples was done by a ready-to-use, TRIzol^{TM} reagent from Thermo Fischer Scientific; USA. This reagent is a solution of phenol and guanidinium isothiocyanate that denatures protein and solubilizes cellular materials. Chloroform addition causes phase separation into a protein containing organic phase, a DNA containing interface and an containing RNA aqueous phase (16). RNA extraction was accomplished by performing the following steps:

Sample Lysis: Frozen blood samples in tubes containing TRIzol^{TM} were left to thaw at room temperature and then were homogenized by pipetting up and down several times.

Three phase's separation: A volume of 200 µL of chloroform was added to the lysate and mixed by vortexing. All mixes were incubated for 5 minutes then centrifuged for 10 minutes at 12,000 rpm. After centrifugation, the mixture was separated into three phases: a lower organic phase, an interphase, and a colorless upper aqueous phase. RNA was separated in the aqueous phase, which was then transferred to a new tube.

RNA precipitation and washing: A volume of 500 µL of isopropanol was added to the aqueous phase and incubated for 10 minutes, then centrifuged for another 10 minutes at 12000 rounds per minute (rpm). Total RNA was precipitated in the form of a white gel-like pellet at the bottom of the tube. The supernatant was discarded, and a volume of 500 µL of 70% ethanol was added and vortexed briefly to wash the isolated RNA. The mixture was then centrifuged for 5 minutes at 10000 rpm and after that; ethanol was aspirated to allow the pellet to air-dry.

RNA solubility: After drying, the pellet was rehydrated in 100 µL of nuclease-free water then incubated in a water bath at 55–60°C for 10–15 minutes.

Estimation of RNA concentration by fluorescence method: Quantus Fluorometer (Promega, USA) was used to detect the concentration of extracted RNA. A volume of 1 µl of RNA was mixed with a volume of 199 µl of diluted Quantifluor Dye followed by 5 minutes of incubation at room temperature in a dark place. RNA concentration values were then estimated by the Quantus fluorometer in units of ng /µL. RNA purity was also measured, and the purity was estimated by measuring the ratio of optical density (OD) of DNA and protein absorption at wavelength 260 / 280nm. The accepted ratio for purity of RNA is 1.7- 2.

Primers: Two primers were used for estimation of IL-22 gene expression (Table 1), a specific primer for interleukin 22 gene (IL-22) from Ikeuchi *et al.* (10) and another primer specific for testis enhanced gene transcript gene (TEGT) or BAX inhibitor 1 gene which was used as a reference gene from Fu *et al.* (9). These were supplied by Macrogen Company in a lyophilized form. Each of these primers was dissolved in an appropriate amount of nuclease-free water to yield a stock solution with a concentration of 100 pmol/µL. A working-solution with a concentration of

10pmol/µL was made by addition of 10µl of the stock solution (stored frozen at -20 C) to nuclease-free water (90µl). Primers sequences and their annealing temperature (Tm) are mentioned in the following table (Table 1).

Table 1. Oligonucleotides of Primers used for TEGT and IL-22 genes

Primer Name	Sequence	Tm (ºC)
TEGT-F	5`-TGCTGGATTTGCATTCCTTACA-3`	60
TEGT-R	5`-ACGGCGCCTGGCATAGA-3`	
INTR22-F	5`-ACAACACAGACGTTCGTCTCATTG-3`	
INTR22-R	5`-GAACAGCACTTCTTCAAGGGTGA-3`	

Gene expression protocol one-step RT-qPCR method was performed using GoTaq®1-Step RT-qPCR System (Promega, USA). This method allows the use of a single reaction for the reverse transcription step in which complementary DNA (cDNA) is synthesized from messenger RNA (mRNA) and the qPCR steps in which cDNA is amplified. The RNA templates and all reagents were thawed, and each solution was mixed gently before mixing the reaction components.

The reaction volume for this procedure was 10 µL according to the instructions of the manufacturer (Promega, USA): 5 µL master mix, 0.25 µL RT mix, 0.25 µL $MgCl_2$, 0.5 µL of each primer, 1 µL RNA and 2.5 µL nuclease-free water (Table 2).

Table 2. Reaction mixture for qPCR

Component	Volume (µL)
qPCR Master Mix (X)	5
RT Mix (X)	0.25
$MgCl_2$ (25mM)	0.25
Forward Primer (µM)	0.5
Reverse primer (µM)	0.5
Nuclease free water	2.5
RNA (ng/µL)	1
Total volume	10

MIC qPCR Cycler (BioMolecular System, Australia) was used to perform RT–qPCR run by applying the following cycling conditions following several; optimization steps: 15 minutes for synthesis of cDNA at 37˚C (1 cycle), 5 minutes for initial denaturation at 95˚C (1 cycle), followed by 40 cycles of 20 seconds for denaturation at 95˚C, 20 seconds for annealing at 60˚C and 20 seconds for extension at 72˚C (Table 3).

Table 3. The program of TEGT and IL-22 genes.

Step	Stage	Temp. (˚C)	Duration	Cycle no.
Hold Step	Reverse transcription	37	15 min	1
	Hot start	95	5 min	
Amplification	Denaturation	95	20 sec	40
	Annealing	60	20 sec	
	Extension	72	20 sec	
Melt on green	Melting curve	72 -95	0.3˚C/sec	1

The real-time cycler software was used to calculate the detected threshold cycle (C_t) for each sample, and then mean values were calculated for duplicate samples. Expression levels were normalized against the housekeeping gene TEGT and gene expression levels were calculated according to the relative quantification method by Livak and Schmittgen (2001) in which results are reported as a fold change in expression levels (14). Three equations were applied which summarize the optimal calculations used to find folding for any tested gene and compare it among groups; these are:

Folding $=2^{-\Delta\Delta CT}$

$\Delta\Delta CT = \Delta CT_{Treated} - \Delta CT_{Control}$

$\Delta CT = CT_{gene} - CT_{House\ Keeping\ gene}$.

Statistical analysis: ANOVA statistical analyses were performed using GraphPad Prism 8 for Windows. Differences were considered to be statistically significant if *P* values were < 0.05.

RESULTS AND DISCUSSION

Subjects: The age and gender distribution were similar among the patients' groups and control group with *P* values of >0.05. The mean body mass index (BMI) was 31.15±5.98 for patients and 28.47±5.97 for the control group (P= 0.296, no significant difference). A BMI of more than 30 indicates obesity which is related to slight variations in the thyroid gland function through different mechanisms (8, 23).

Thyroid evaluation: patients with newly detected autoantibodies against the thyroid gland and not receiving levothyroxine therapy (group A) had a significantly higher (P < 0.01) mean TSH and lower T3, T4 serum concentrations than the levothyroxine-treated autoimmune hypothyroidism patients (group B) and the control group, whereas TSH, T3

and T4 mean serum concentrations did not differ significantly (P>0.05) between the latter groups (group B and control group) considering patients diagnosed with autoimmune hypothyroidism had already normal thyroid hormones levels in their serum due to levothyroxine replacement therapy; therefore, TSH levels produced by the pituitary gland remained within the normal range (Table 4). These results indicate that patients in subgroup A are at risk of developing autoimmune hypothyroidism.

Table 4. Mean serum concentrations of the thyroid gland evaluation hormones

	TSH ± SD (IU/mL)	T3 ± SD (nmol/L)	T4 ± SD (nmol/L)
Group A (n=13)	**6.3 ± 3.35**	**1.14 ± 0.5**	**60.75 ± 23.43**
Group B (n=15)	**1.91 ± 1.06**	**1.95 ± 0.54**	**101.2 ± 24.44**
Control group (n=12)	**2.64 ± 0.91**	**2.13 ± 0.53**	**100.8 ± 18.67**
P*-value	**a=0.001 b=0.001 c=0.072**	**a=0.001 b=0.001 c=0.889**	**a=0.001 b=0.001 c=0.395**

***P-value (ANOVA test)** where a: group A vs. group B; b: group A vs Control; c: group B vs Control.

In group A, 38.4 % of patients (n=5) were positive for anti-TG with a median of 380.4 IU/mL (208.7 - 2998.9 IU/mL) and 92.3 % of them (n=12) were positive for anti-TPO with a median of 301.9 IU/mL (41.6 – 960.7 IU/mL). These concentrations had no significant difference with group B in regards to anti-TG and anti-TPO concentration (*P*=0.5757, 0.4814 respectively). Group B had 53.3 % anti-TG positive patients (n=8) with a median of 282.9 IU/mL (134.3- 2661.0 IU/mL) and 93.3 % anti-TPO positive (n=14) with a median of 437.5 IU/mL (65.3 - 1018.4 IU/mL). Both group A and B showed a highly significant difference (P<0.01) with the control group in which the highest detected concentrations of autoantibodies were 35.4 IU/mL for anti-TG and 20 IU/mL for anti-TPO. These concentrations are considered negative according to the manufacturer's instructions. The remaining control group concentrations were either below these values or undetectable.

Gene expression of IL-22: Extraction of total RNA was successfully achieved with concentrations ranges of 10 – 211 ng/ µl and purity ranges of 1.75 – 1.95 . Synthesis of cDNA was carried out as the initial step in RT-qPCR followed by amplification of target genes. Gene expression calculations involved the analysis of double delta Ct to assess the IL-22 gene expression, in which the housekeeping gene TEGT was a reference gene. The amplification was registered as a value of Ct (Figures:1,2).

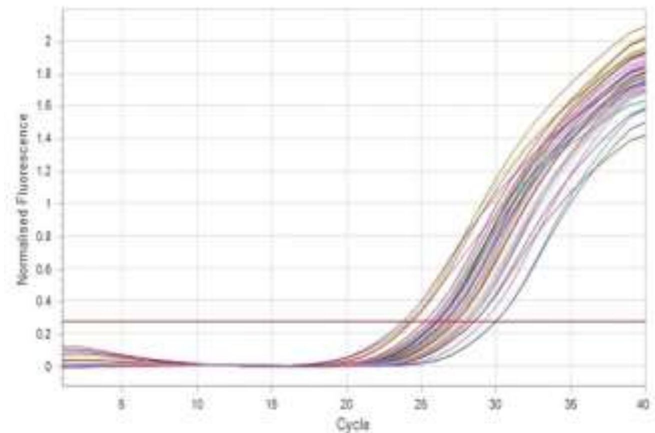

Figure 1. Amplification curve for TEGT reference gene (Ct value)

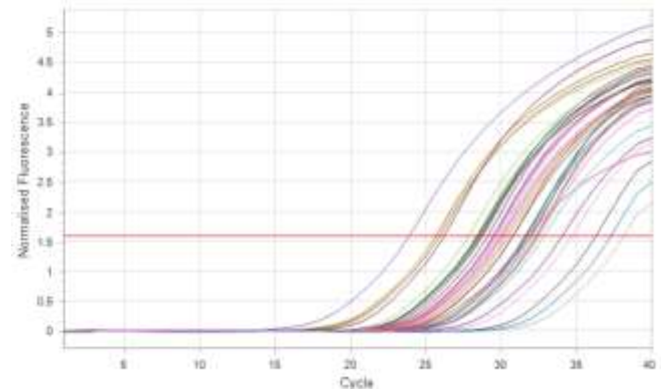

Figure 2. Amplification curve for IL-22 gene (Ct value)

The lower Ct value indicated that higher copies of the target gene were present and vice versa. In terms of gene expression, high Ct values indicated low gene expression and low Ct values indicated a high gene expression (14). The $2^{-\Delta\Delta Ct}$ value was calculated to obtain the relative fold change for expression of IL-22 gene. Calculations involved, First, the mean of Ct values for the housekeeping gene (TEGT) and the gene being tested (IL-22) in patients and controls were calculated. Four Ct values were obtained from these calculation which are; patients' Ct value for IL-22 gene (TP), control subjects' Ct value for IL-22 gene (TC), patients' Ct value for TEGT (HP) and

control subjects' Ct value for TEGT (HC). Second, values of ΔCt for patients (ΔCtP) was obtained by calculating the difference between TP and HP (TP-HP), while control subjects' (ΔCtC) was obtained from calculating the difference between TC and HC (TC-HC). Third, Double Delta Ct value (ΔΔCt) was obtained by calculating the difference between ΔCtP and ΔCtC (ΔCtP-ΔCtC).

Melt curve analysis: To ensure the specificity of the reaction, a melt curve analysis was performed on PCR products at the end of the PCR cycles to confirm the primers' annealing specificity. The presence of a single sharp peak on the melt curve indicates one PCR product thus, ensuring the results' reliability (22) (Figures 3,4).

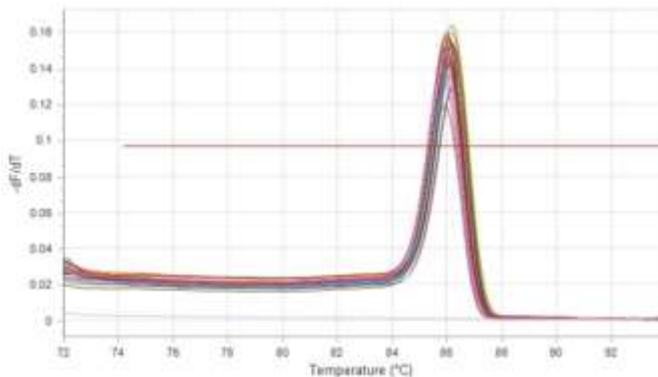

Figure 3. Melting curve analysis of TEGT gene

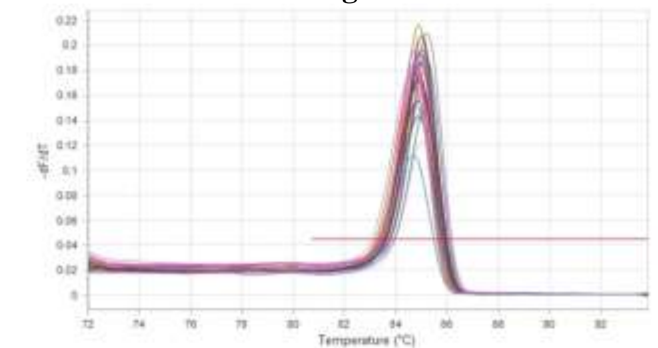

Figure 4. Melting curve analysis of IL-22

The ΔCt mean of IL22 mRNA showed a significant decrease in patients in group A when compared to patients in group B while this decrease was not significantly different than the Ct of the control group (Table 5). In terms of a relative expression, the $2^{-\Delta\Delta Ct}$ mean of patients in group A yield the highest folds of IL-22 expression (3.62 ± 4.35) (Table 5, Figure 5) with great variability between individuals. This increase in folds of expression had a significant difference compared to patients in group B who are receiving treatment for hypothyroidism and a non-significant difference compared to the control group.

Table5. ΔCt and fold mean values ± SD

	ΔCt Mean ± SD	Folds mean ± SD
Group A (n=13)	**1.94±1.64**	**3.69 ± 4.32**
Group B (n=15)	**4.01±2.49**	**0.92 ± 0.77**
Control group (n=12)	**2.95±1.95**	**1.85 ± 1.64**
P*-value	**a=0.017 b=0.17 c=0.24**	**a= 0.022 b= 0.179 c= 0.066**

***P-value (ANOVA test)** where a: group A vs. group B; b: group A vs Control; c: group B vs Control.

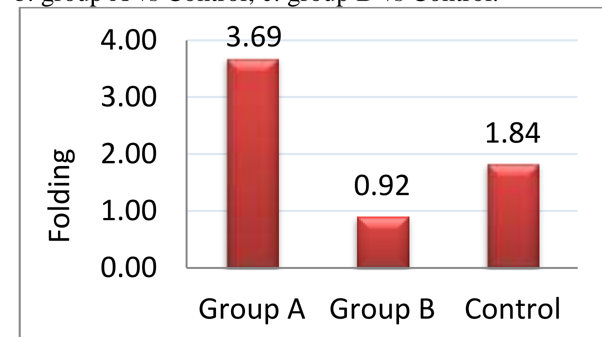

Figure 5. Folds of IL-22Gene Expression

This can be explained by the fact that patients already diagnosed with autoimmune hypothyroidism and established thyroxine replacement therapy have gotten a long path in duration of the immune response against the thyroid gland. The eventual destruction of the thyroid gland, which resulted in hypothyroidism, could have caused the immune balance to be restored and levels of IL-22 returned to normal state. On the other hand, patients with newly detected autoantibodies may have an immune system imbalance or dysregulation and could be at high risk of developing autoimmune hypothyroidism and require follow up due to the realization of epidemiological studies that TPO-Ab may be present even in apparently healthy individuals, but after a period of seven to nine years, they will develop autoimmune hypothyroidism (20). In addition, Il-22 has been addressed as having a key role in the development of several autoimmune diseases including AITD as reported by previous studies (11). The results agree with Ruggeri *et al.,* who also found that serum IL-22 levels

were higher in HT patients who were newly diagnosed and untreated compared to healthy controls (16). Moreover, Bai *et al.* found that circulating Th22 cells which produce IL-22 are detected in significantly higher levels in HT patients as compared with the healthy control group and the GD patient group, and was positively correlated with the serum IL-22 level and anti-TPO antibodies titer (3). This increase of IL-22 in autoimmune diseases may serve as a promising therapeutic target as reported by a recent preclinical study which indicated that IL-22 can be neutralized with specific antibodies and thus, inhibiting its action (24)

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