University of Kerbala

College of Pharmacy

Department of Pharmacology and Toxicology

The Impact of Deiodinase-2 Polymorphisms on the Therapeutic Response of Levothyroxine of Hypothyroidism Female Patients in Kerbala Province

A Thesis

Submitted to the Council of College of Pharmacy / University of Kerbala as Partial Fulfillment of the Requirements for the Degree of Master of Science in Pharmacology and Toxicology

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بِسْمِ اللهِ الرَّحْمَنِ الرَّحِيمِ

(إِنَّمَا يَخْشَى اللَّهُ مِنْ عِبَادِهِ الْعُلَمَاءُ)

صدق الله العلي العظيم

سورة فاطر جزء من الآية 28

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Dedication

To the cornerstone of scientific research,

To their curiosity,

To their pursue for knowledge,

To the wonderful species,

To all humankind.

Muntadher

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Chapter One: Introduction

List of Abbreviations	
Abbreviations	***Full-Text***
T3	Tri-iodothyronine
T4	Tetra-iodothyronine
TSH	Thyroid-stimulating hormone
NHANES	National Health and Nutrition Survey
TRH	Thyroid-releasing hormone
TBII	thyrotropin binding inhibitor immunoglobulins
LDL	low-density lipoprotein
TG	triglycerides
TPO	thyroid peroxidase
ATP	adenosine triphosphate
IR	insulin resistance
HOMA IR	Homeostatic Model Assessment for Insulin Resistance
TC	total cholesterol
HI	Hyperinsulinemia
HOMA	Homeostasis model assessment
VTE	venous thromboembolism
PCOS	polycystic ovarian syndrome
rT3	reverse triiodothyronine
T2	Diiodothyronine
D1	Deiodinase type 1
D2	Deiodinase type 2
D3	Deiodinase type 3
BAT	Brown adipose tissue
SNP	Single Nucleotide Polymorphism

EDTA	ethylene diamine tetra acetic acid
ELISA	enzyme-linked immunosorbant assay
BMI	Body Mass Index
PCR	Polymerase Chain Reaction
NCBI	National Center for Biotechnology Information
BLAST	Basic local alignment search tool
cAMP	cyclic Adenosine Mono Phosphate

Abstract

Background: There is a clinical observation in the local population of hypothyroidism patients that many patients still have symptoms and disease-complaints even after they treated with levothyroxine as a replacement therapy. Deiodinase type 2 enzyme plays a central role in the conversion of thyroxin to the active form triiodothyronine . This study investigates the effect of rs225014; 274 T>C and rs225017; A>T single nucleotide polymorphisms in deiodinase type II gene on the clinical response to levothyroxine replacement therapy in a sample of hypothyroidism patients in Kerbala province.

Methodology: In this cross –sectional study, one hundred fifty Iraqi female patients with primary hypothyroidism of the age of 40 or older, who were treated with levothyroxine were recruited. Thyroid hormones (free and total T4, free and total T3, revers T3 , diiodothyrionine) were assessed. The genetic analysis to detect rs225014 SNP was done using the tetra primers amplification refractory mutation system-polymerase chain reaction technique, to detect rs225017 SNP; direct sequencing using Sangar protocol for the specific amplified products was done.

Results: The genotypes distribution of rs225014; 274 T>C SNP was 22 (14.66%), 19 (12.66%) and 72 (72.66%) as TT, TC and CC, respectively. Total T4 was significantly higher in TC carriers than in CC carriers, while there were no significant differences in the levels of TSH, the free and total T3, the free and total T4 and the levothyroxine dose in TT, TC and CC groups of patients. The TC group also had significantly higher fasting serum insulin and homeostatic model assessment for insulin resistance. Regarding 130 female patients with rs225017; A>T SNP, the genotypes

distribution was 120 (92.3%), 9 (6.9%) and 1 (0.8%) as AA, AT and TT, respectively. There was a significant difference in the T2 level and T3/T4 molar ratio among the three groups while there was no significant association between this SNP and free and total T3, free and total T4, TSH, rT3, and the levothyroxine dose.

Conclusions: Since the rs225014 and rs225017 SNPs of DIO2 gene are not associated with the most of the thyroid hormones levels, they could not affect the response to levothyroxine treatment, although rs225014 SNP cannot be excluded from being related to the hypothyroidism because the homozygous mutant type CC of it is the most frequent genotype in present sample of Iraqi hypothyroidism patients.

1. Introduction

1.1 Thyroid Gland

The thyroid is one of the largest endocrine glands in the body. It is situated between the fifth cervical and the first thoracic vertebrae at the front of the neck, beneath the voice box [1]. It consists of right and left lobes that are joined by a small, central isthmus as shown in figure (1-1)

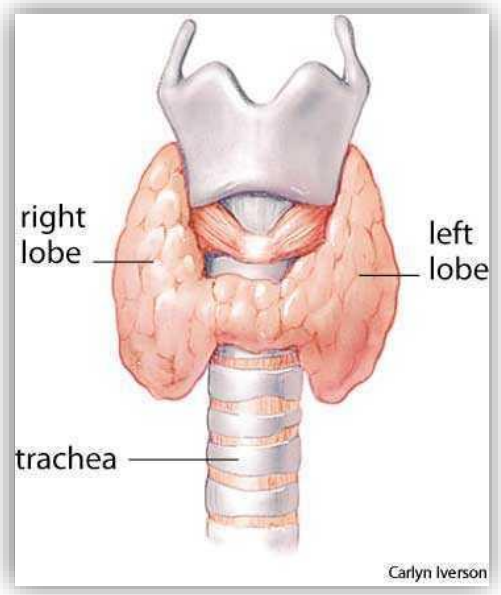


Figure 1-1: Location of the thyroid gland [2]

The functional units of the thyroid are follicles; which consist of a central colloid core surrounded by a single-layered epithelium resting on a basal lamina. The colloid consists almost entirely of iodothyroglobulin, which is an iodinated glycoprotein-stored form of the active thyroid hormones (tri-iodothyronine (T3), and prohormone tetra-iodothyronine or thyroxine (T4)). Sufficient iodothyroglobulin is stored

within follicles to regulate the metabolic activity of the body for up to 3 months [4,5].

Less than 1% of the total plasma T4 and T3 are free, while around 95% of the thyroid hormones released are linked to plasma proteins [5]. T3 is mostly created by the extrathyroidal deiodination of T4, with the thyroid producing only 20% of it directly [6].

The thyroid gland is considered the master gland of metabolism and its hormones are responsible for controlling the body's metabolic rate, upregulating the growth of the body, especially the development of neurons, thyroid gland also has a role in thermogenesis, reproduction, and defense against viruses and free radicals. The anterior pituitary releases Thyroid-stimulating hormone (TSH), which controls the thyroid gland and its hormones [7].

Among thyroid gland disorders are hyperthyroidism, thyroid nodules, and thyroid cancer but the most common disease is hypothyroidism [8].

1.2 Hypothyroidism

Hypothyroidism is a chronic disease and one of the most common endocrine disorders caused by the underproduction of thyroid hormones [9]. Infertility, cardiovascular illness, neurological and musculoskeletal complaints, and other conditions can result from untreated or improperly managed hypothyroidism because thyroid hormones have an impact on many areas of metabolism and growth [11].

1.2.1 Epidemiology

Hypothyroidism prevalence depends on many parameters, it is more common in white people, obese (BMI>30), and is age-

dependent (the National Health and Nutrition Survey (NHANES III) confirmed that both TSH level and the presence of antithyroid antibodies are increased with age and the prevalence of hypothyroidism in females (5.9%) more than in males (2.3%) [11].

In the United States, 4.6% of the population had hypothyroidism (0.3% overt and 4.3% subclinical). In Europe, the prevalence of total, overt, and subclinical hypothyroidism in females were 6.40%, 0.80%, and 5.86%, respectively, whereas in males they were 3.37%, 0.30%, and 3.45%, respectively [12]. In Arab countries, the prevalence of hypothyroidism in Libya was 6.18%, while in Saudi Arabia subclinical hypothyroidism was 11.1% and overt hypothyroidism was 4% [13]. In Iraq, a study conducted in Kirkuk province found that 22.7% of the population had hypothyroidism [14] while in Kurdistan, 4% of females had hypothyroidism [15].

1.2.2 Etiology

A normal thyroid gland uses iodine from the diet to synthesize thyroid hormones (T4 and T3). The thyroid gland and its products are under the pituitary gland control through its hormone TSH by a negative feedback mechanism, if there is a deficiency in T4 and T3 the pituitary gland will respond by releasing TSH to stimulate the thyroid gland. The hypothalamus is responsible for stimulating the pituitary gland through thyroid-releasing hormone (TRH). This hypothalamus-pituitary-thyroid axis is responsible for maintaining the normal blood levels of thyroid hormones [16].

If there is a failure in the thyroid gland itself, it will result in primary hypothyroidism, while a failure in the pituitary gland will result

in secondary hypothyroidism, and tertiary hypothyroidism comes from the failure of the hypothalamus [17].

The causes of primary hypothyroidism are acquired, congenital, and pharmacological. Iodine deficiency, which affects 28.9% of the global population, is the most prominent underlying cause of acquired hypothyroidism in general [10]. In areas with sufficient iodine sources, autoimmune thyroiditis, primarily Hashimoto's thyroiditis, is the most commonly cause of acquired hypothyroidism. The second frequent cause of acquired hypothyroidism is thyroid ablation either by radiation or thyroidectomy in case of thyroid nodules, thyroid cancer, or Graves' disease, in which 90% or more of the gland must be removed leading to hypothyroidism [18].

On the other hand, congenital hypothyroidism comes from three causes: aplasia when the child is born without a thyroid gland, hypoplasia when the thyroid gland is not completely formed, and thyroid ectopy when the gland is not in its right place (ectopic thyroid) [19].

The pharmacological causes of hypothyroidism are due to the effects of some drugs such as amiodarone, lithium, interferon-alpha, and tyrosine kinase inhibitors were found to cause hypothyroidism in 36% to 71% of patients [20].

1.2.3 Pathophysiology

The pathophysiology of hypothyroidism is complex and involves several etiologic factors. The inability of the thyroid gland to produce physiologically necessary levels of T4 and T3 is known as dyshormonogenesis. Defects in iodine trapping, organization, coupling, transport, thyroglobulin synthesis, or deiodination may be the cause of this condition. An organification deficiency comprises structural changes

to the amino acid sequence of the iodine acceptor molecule thyroglobulin as well as qualitative or quantitative abnormalities of the thyroid peroxidase system. The development of hypothyroidism may also be influenced by antithyroperoxidase antibody's inhibition of peroxidase enzyme activity. Autoantibodies to sodium-iodide symporter protein like in autoimmune thyroiditis inhibit iodide uptake and transport [21].Additionally, decreased or absent TSH receptor response to TSH causes inadequate iodide uptake and then, decreased thyroid hormone synthesis. This TSH resistance may cause by the blocking effect of thyrotropin binding inhibitor immunoglobulins (TBII), or molecular defects affecting the transmission of the TSH stimulatory signal [22].

1.2.4 Clinical and Biochemical Features of Hypothyroidism

The signs and symptoms of hypothyroidism are not specific and a patient may not have all the clinical features. The most typical signs and symptoms are generally weakness, irregular menstruation, lack of concentration, cold sensitivity, constipation, hair loss, edema, weight gain, goiter, cognitive impairment, and delayed relaxation phase of deep tendon reflexes [23].Bradycardia, low voltage, and flattened T-waves are some potential electrocardiography findings. Hemodynamic instability, coma, pericardial effusion, and pleural effusion are all possible signs of severe hypothyroidism [24].

Hypothyroidism symptoms vary with age, infants and children may present more often with lethargy and failure to thrive, while in the elderly cognitive decline is a common manifestation [25].

Carpal tunnel syndrome, sensorimotor polyneuropathy, elevated rates of depression and anxiety, and psychomotor slowness are some neurological symptoms. Skin xerosis, reduced sweating, skin thickening,

brittle hair, hair loss, loss of the lateral brows (Queen Anne sign), and vitiligo are all dermatological signs [20].

The gastrointestinal manifestations are dyspepsia, gastroesophageal reflux, and constipation. Sexual changes, in women, include oligomenorrhea, amenorrhea, and menorrhagia, while in men there is a decrease in testosterone hormone and hypogonadism because hypothyroidism is associated with hyperprolactinemia [26].

Biochemical tests may show low or normal TSH with low T3 and T4 in case of secondary hypothyroidism while raised TSH with low T3 and T4 in case of primary hypothyroidism [27]. Increased levels of C-reactive protein, hyperprolactinemia, hyponatremia, creatine kinase, low-density lipoprotein (LDL) cholesterol, elevated triglycerides (TG), normocytic anemia, and proteinuria may be present in hypothyroid patients [28].

1.2.5 Diagnosis

Both a clinical examination and biochemical tests are used to determine the hypothyroidism diagnosis. The clinical examination depends on the signs and symptoms of the disease and the physical examination of the thyroid gland and its size. The delayed relaxation phase of the deep tendon reflexes which is a classic physical finding can be detected, also myxedema is sometimes seen [29].

Biochemical tests include measurement of TSH level which is a very sensitive and reliable test but must be done with other thyroid tests. The normal range of TSH is 0.5 to 5.0 mIU/L [30], but in the elderly, the upper limit goes up to 7.9 mIU/L, high TSH value indicating primary hypothyroidism while if it's normal or low that indicates secondary hypothyroidism.

Secondly, total T4 is used but not free T4, because patients may have protein abnormalities or taking some drugs that may cause unpredictably false free T4 [31].

On the other hand, T3 is not a sensitive test to thyroid hypofunction; because T3 value remains normal until severe hypothyroidism ensues, starvation and many drugs can result in low serum T3 value even in euthyroid individuals.

Also, the serum thyroid peroxidase (TPO) antibodies can be determined to investigate autoimmune-related hypothyroidism [32].

1.2.6 Complications

In general, thyroid hormones regulate metabolic rate, influencing adiposity, controlling core body temperature, appetite, and sympathetic activity. Weight gain and abnormalities of serum glucose and triglycerides are linked with high TSH levels, also, hypothyroidism has been related to higher blood pressure [33].

Thyroid hormones boost the use of adenosine triphosphate (ATP), which speeds up the anabolic and catabolic pathways in the macronutrient metabolism, such as accelerated protein turnover and lipolysis/fatty acid oxidation. One study discovered that, regardless of insulin sensitivity, metabolic parameters, or blood pressure, central fat storage is directly correlated with elevated serum TSH levels [34].

Triiodothyronine promotes the translocation of the glucose transporter 4 (GLUT 4) to the plasma membrane in skeletal muscle and adipose tissue, which is associated with improved glucose tolerance; hence, hypothyroidism has long been associated with insulin resistance (IR) [36,37]. One study found a significant positive correlation between

TSH and insulin as well as TSH and Homeostatic Model Assessment for Insulin Resistance (HOMA IR) in the female population suffering from both subclinical hypothyroidism and overt hypothyroidism [37]. HOMA-IR was associated with low free T3 and low total T3 levels in non-diabetic individuals [38].

Thyroid hormones potently promote hepatic cholesterol absorption and hepatic cholesterol excretion as bile acid; as a result, low-density lipoprotein (LDL)-c accumulates in hypothyroid individuals' circulation. Patients with subclinical hypothyroidism have higher levels of total cholesterol (TC), LDL cholesterol, and even perhaps TG [40, 41].

Patients with moderate and subclinical hypothyroidism have been found to have a number of significant cardiovascular risk factors, such as diastolic dysfunction, increased arterial stiffness, endothelial dysfunction, and an increase in systemic vascular resistance [41]. Patients are more likely to develop chronic cardiac disease when their plasma TSH levels are higher than 10 mU/L. Approximately 20% of women who have thyroid dysfunction have also diastolic hypertension compared with 3.4% of euthyroid women [42]. Also, a study conducted in Taiwan indicated a significant association between hypothyroidism and the increased risk of venous thromboembolism (VTE) [43].

Reproduction and fertility are directly affected by thyroid hormones [44]. Hypothyroidism is a common cause of infertility but it can be easily managed by levothyroxine administration, one study conducted in India found that 76.6% of infertile hypothyroid women conceived after receiving levothyroxine [45]. In addition, hypothyroidism makes ovarian morphology poly-cystic, hence hypothyroidism should be excluded before making a diagnosis of the polycystic ovarian syndrome (PCOS) in any woman [46].

1.3 Levothyroxine

Levothyroxine is a synthetic thyroid hormone that resembles the natural endogenous hormone in structure and action, levothyroxine is indicated for primary, secondary, and tertiary hypothyroidism. It is also used for euthyroid goiter, multinodular goiter, thyroid nodules, and thyroiditis [47].

From the seventies of the last century, levothyroxine was used as an oral therapy, and since then, it convert hypothyroidism from a morbid disease to a successively managed one [49,50].

Thyroid hormone replacement aims to neutralize TSH value and correct the symptoms of the deficiency, levothyroxine is successful at meeting these criteria [50].

1.3.1 The Optimal Dose Determination

The optimal dosing window is 1.6-1.8 mcg/kg/day for approaching a normal TSH value [51].

Many factors may contribute to determining thyroxine dose requirement, primarily: the amount of residual thyroid function retained by the patient, lean body mass of the patient, and the target of TSH that is wanted, to a lesser extent, patient age, patient sex, and menopausal status, pregnancy and gastrointestinal status.

The thyroidectomized patients require more doses than autoimmune thyroid diseases like Hashimoto's thyroiditis because in the latter case there are residual functions left [52]. Because adipose tissue is less responsive to levothyroxine than muscles, lean body mass is more preferred than body weight in predicting levothyroxine requirement [50].

Because protein-bound thyroid hormones are lost through

urination in nephrotic syndrome patients, they require higher dosages of levothyroxine [53].

Replacement therapy is different according to the patient's condition, like in pregnancy, levothyroxine requirements need to increase by 30%-50% because the fetal thyroid gland starts functioning only after 12–14 weeks of gestation, and the plasma volume will increase so the thyroxine pool must increase accordingly [54]. While in the elderly population the replacement dose decreases with age because of a decrease in lean body mass and thyroxine degradation [55].

1.3.2 Mechanism of Action

Levothyroxine is the L-form of the endogenous thyroid hormone T4, which is a prohormone that deiodinases enzymes convert to the active hormone T3. T3 controls differentiation and metabolic rate by binding to thyroid receptors (TR) and then activating or repressing specific genes [56].

Thyroid hormone receptors (TRs) are nuclear receptor superfamily members. There are two isoforms of the thyroid receptor, TRα, and TRβ. TRα is expressed in the brain, heart, and skeletal muscle, while TRβ is expressed in the brain, retina, inner ear, kidney, liver, and lung [57].

After the binding of T3 to the thyroid receptor, conformation changes will occur and then the complex bind to the DNA response element as a heterodimer in a specific orientation. Binding of the complex to DNA causes either expression or repress of specific genes, also in the absence of any ligand, TR can bind to DNA but this binding represses or silences basal target gene transcription [58], T3 can cause an increase in the left ventricular contractility because it stimulates the

transcription of myosin heavy chain α and inhibits the expression of myosin heavy chain β [59].

1.3.3 Levothyroxine Pharmacokinetics

Absorption of Levothyroxine mainly occurs in the small intestine with bioavailability of 80% decrease with food to approximately 64%. Levothyroxine volume of distribution is equivalent to the volume of extracellular fluid. The major metabolic pathway for thyroxine is deiodination by deiodinase enzymes, mainly in the liverT4 convert to T3 which has the active hormonal activity by outer ring deiodination, and to reverse triiodothyronine (rT3) which is the inactive form by inner ring deiodination and both T3 and rT3 convert to diiodothyronine (T2) which is the end product of thyroid hormones. T4 has a half-life of 7.5 days in hypothyroid patients and 6.2 days in euthyroid people, whereas T3 has a half-life of approximately 1.4 and 1.0 days in hypothyroid patients and euthyroid volunteers, respectively. Metabolites are primarily eliminated via the kidneys [47].

Table 1-3: Summary of Levothyroxine Pharmacokinetics[47]

Pharmacokinetic Characteristic	Description
Main site of absorption	Small intestine (jejunum and ileum)
Bioavailability	70–80 % in euthyroid person; may be slightly higher in hyperthyroid patients
Tmax	2–3 hours
Vd	11–15 L
Protein binding	T_4 >99.9 % T_3 = 99.8 %
T1/2	T_4 = 6.2 and 7.5 days in euthyroid and hypothyroid patients, respectively T_3 = 1.0 and 1.4 days in euthyroid and hypothyroid patients, respectively
CL	T_4 = 0.055 and 0.038 L/h in euthyroid and hypothyroid patients, respectively

Vd = Volume of distribution; L = Litres; CL = Clearance

1.3.4 Food-Drug Interactions

Food may affect the bioavailability of levothyroxine like soy-containing foods, papaya, grapefruit, and coffee. The fiber-rich diet also affects bioavailability because fiber and bran bind to thyroxine and then decrease its absorption [52].

Drugs like cholestyramine, colestipol, colesevelam, sucralfate, aluminum hydroxide, proton-pump inhibitors, raloxifene, orlistat, laxatives and antacids containing magnesium interact with levothyroxine and then decrease its bioavailability. Supplements like calcium and iron decrease levothyroxine absorption while vitamin C increases it [52,56,62].

1.4 Deiodinase Enzymes

Enzymes are in charge of accelerating biological reactions at speeds appropriate for any living system's regular operation, growth, and reproduction. Iodothyronine deiodinases are members of the oxidoreductases family that contain selenocysteine in their active site and are involved in the local and peripheral regulation of thyroid hormones. Deiodinase type 1 (D1) and deiodinase type 2 (D2), which convert T4 into the physiologically active hormone (T3) and remove rT3, are two of the three types of deiodinases that are encoded by distinct genes. While deiodinase type 3 (D3) converts T3 to T2 and T4 to rT3 as shown in Figure 1-2. [61]

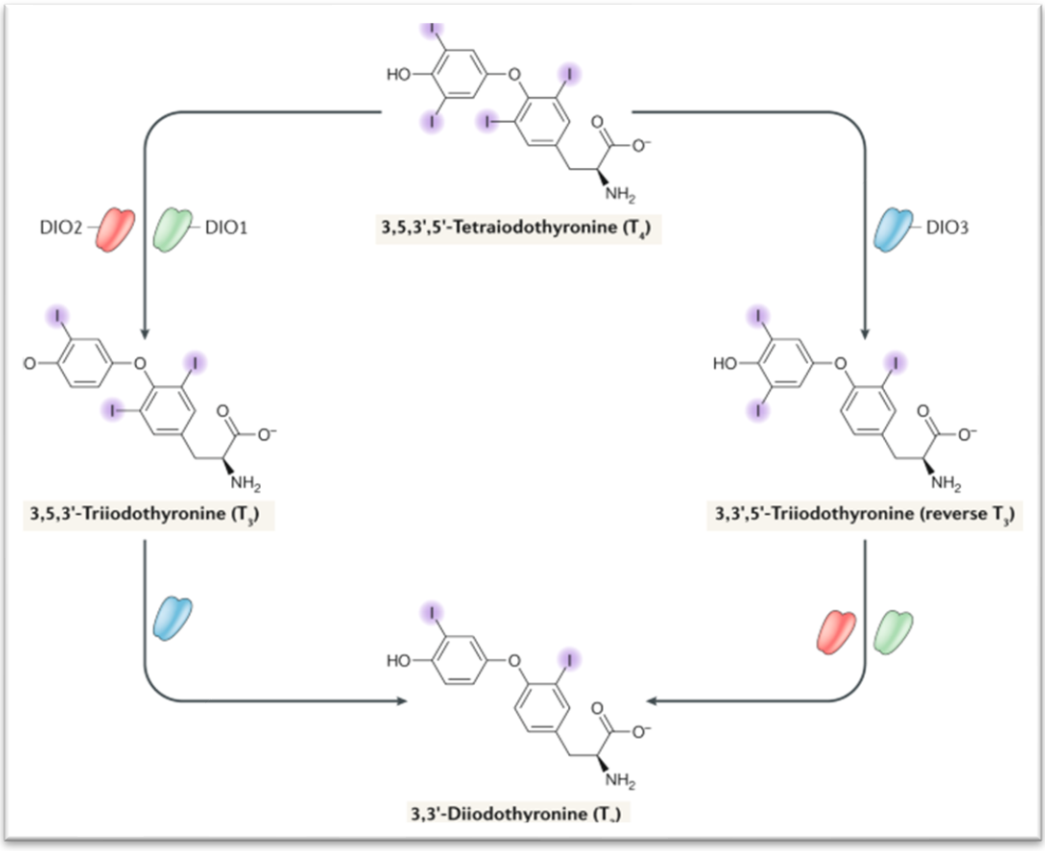


Figure1- 2: Types and roles of deiodinases enzymes [62]

Deiodinases are expressed differently in different tissues; D1 is found in the liver, kidney, and thyroid gland, D2 in the central nervous

system, pituitary, skeletal muscle, thyroid, heart, bone, and brown adipose tissue, and D3 is mostly found in the placenta [65,66].

1.4.1 Deiodinase type 2

Deiodinase type 2 is an enzyme found in the plasma membrane of particular cells. Its function is to convert T4 to T3 by deiodination at the phenolic ring (outer ring deiodination) independent of circulating T3 [65].

Deiodinase-generated T3 plays a significant role in several biological systems such as the pituitary and hypothalamic feedback mechanism, retina development, brown adipose tissue (BAT) development, bone maturation, and muscle regeneration [66].

Studies on knock-out mice have been developed to better understand the effects of the enzyme. It has been discovered that these mice have isolated hypothyroidism in tissues that depend on DIO2-catalyzed T4 to T3 conversion. They also have elevated TSH and are unable to maintain a normal body temperature after being exposed to cold temperatures [67].

1.4.2 Deiodinase type 2 Gene Polymorphisms Effects

The Deiodinase type 2 (DIO2) gene, which encodes human D2, is responsible for over 70% of the amounts of circulating serum T3 in humans [68].

The coding region of this single-copy gene is located at position 14q24.3 on the long arm of the 14th human chromosome. Its coding site is separated into two exons by a 7.4-kb intron [69]. Its gene expression rises when there is little T4 in the blood. Also, DIO2 activity is increased

by the different agents that increase cyclic Adenosine Mono Phosphate (cAMP) production like corticosteroids and growth factors [70].

Single Nucleotide Polymorphism (SNP) is a slight variation in the genome of individuals belonging to the same species. These differences contribute to diversity among individuals and, in some cases, can predispose one to disease or impact the response to substances or agents [71].

In general, a significant DIO2 gene polymorphism can affect deiodinase type 2 enzyme catalytic activity and T3 production, also associated with insulin resistance and increased BMI and subsequently with diabetes mellitus type 2 and a bunch of other diseases like mental retardation, hypertension, osteoarthritis, and bipolar disorder [72].

The SNP known as Thr92Ala (rs225014) is a common variant in the general population, and the homozygous mutant prevalence in the European population is 12.9%-14.9% [73].

Recent studies showed that there was a relationship between Thr92Ala and decreased deiodinase activity so there is a necessity in increasing levothyroxine dose by 20% to reach the target TSH in homo mutant patients [76,77], similarly another study found that this mutant type is less efficient in converting T4 to T3 [76], Additionally, it was discovered that persons with the Ala/Ala genotype had D2 activity levels that are half as high as those with the Ala/Thr or Thr/Thr genotypes, and this polymorphism is linked to insulin resistance [77]. A meta-analysis study conducted in the Indian and Brazilian populations showed that patients who were homozygous for Thr92Ala had 4.8% higher HbA1C levels [78]. However, a significant cohort research indicated that neither in the general population nor among those received thyroid hormone

replacement treatment, the Thr92Ala polymorphism was related to changes in thyroid parameters or cognitive function [79]. Other study found that Thr92Ala polymorphism was not associated with thyroid parameters [80].

The second SNP, rs225017 (A/T), is situated at the 3 untranslated region (3UTR) of the DIO2 gene and is linked to insulin resistance in white patients. Patients with the homozygous genotype for both SNPs had a higher HOMA-IR index when compared to patients with other genotype combinations [81].

1.5 Aims of the Study

The present study was designed to investigate:

1. The prevalence of DIO2 genetic polymorphisms (rs225014 and rs225017) in the hypothyroidism females patients of Kerbala province.
2. The possible association between DIO2 genetic polymorphisms (rs225014 and rs225017) with the therapeutic response to levothyroxine of hypothyroidism patients in Kerbala province.

2. Patients, Materials, and Methods

2.1 Study Patients

One hundred fifty females aged 40-74 years had enrolled in this study from August 2021 to March 2022. Patients had attended outpatient clinics for follow-up after they have already been diagnosed with hypothyroidism.

2.1.1 Patients Criteria

2.1.1.A. Inclusion Criteria

Hypothyroid female patients aged 40-74 years received thyroid hormone replacement therapy (levothyroxine) from 4 months and more.

2.1.1.B. Exclusion Criteria

1- Male patients

2- Patients aged under 40 years

3- Patient’s treatment period less than 4 months

4- Patients taking drugs or supplements affecting deiodinases enzymes and/or thyroxine drugs such as propanolol, omeprazole, cimetidine and others

5- Thyroidectomized patients

6- Patients having systemic diseases

2.1.2 Scientific and Ethical Approval

The scientific and ethical committee of the college of pharmacy – at Kerbala University discussed and approved the proposal of the research, the approval number was 2021HU4. All patients signed written

consent with a detailed explanation about the purpose of the study and answered a questionnaire voluntarily.

2.1.3 Study Design

This is a cross-sectional study enrolled 150 participants who were Iraqi female patients diagnosed with hypothyroidism. Blood samples have been taken from overnight fasting patients for biochemical, hormonal, and genetic investigations.

Precautions have been taken in clinical settings to prevent infection of COVID-19.

2.2 Materials

2.2.1 Instruments, equipments and their Suppliers

The instruments that used in the current study are illustrated in Table 2-1.

Table 2-1: Instruments and the manufacturing companies

Types of equipment	Company	Country
Centrifuge	SIGMA	Germany
Cobas e 411	Roche	Germany
Digital camera	Canon	England
Distillator	GFL	Germany
Electrophoresis apparatus	Techinme	England
Hood	LabTech	Korea
Micropipettes	SLAMMED	Japan
PCR machine (Thermocycler)	Verity	USA
Sensitive balance	AND	Taiwan
UV- Trans illuminator	Syngene	England

Vortex mixer	HumanTwist	Germany
Water bath	LabTech	Korea

2.2.2 Chemicals, Kits, and their Suppliers

The chemicals and kits that used in the current study are illustrated in Table 2-2.

Table 2-2: Chemicals, kits, and their producing companies

	Chemicals and Kits	Company	Country
Chemicals	Agarose	Bio Basic	Canada
	Ethanol	SDI	Iraq
	Ethidium Bromide	Intron	Korea
	Nuclease free water	Bioneer	Korea
	TBE buffer	Bioneer	Korea
Hormonal Kits	Diiodothyronine kit	BT Lab	China
	Free Thyroxine kit	Snibe Diagnostic	China
	Free Triiodothyronine kit	Snibe Diagnostic	China
	Insulin kit	Mindray	China
	Reverse Triiodothyronine kit	BT Lab	China
	TSH kit	Snibe Diagnostic	China
	Total Thyroxine kit	Snibe Diagnostic	China
	Total Triiodothyronine kit	Snibe Diagnostic	China

Biochemical Kits	Fasting Serum Glucose kit	Mindray	China
Kits of genetic investigation	DNA Extraction Kit from blood Favor Prep	Favogen	Taiwan
	PCR Master mix Kit	Bioneer	Korea
	DNA ladder Marker (100 bp)	Genome	Korea
	Primers for detection of: DIO2 rs225014 T > C DIO2 rs225017 A > T	Alpha	Canada

2.3 Methods

2.3.1 Samples Collection

Blood samples were drawn from overnight fasting individuals and split into two parts: the first (2ml) was maintained in an ethylene diamine tetraacetate (EDTA) tube for DNA extraction and the second (3ml) was placed in a gel tube to isolate serum for biochemical and hormonal testing.

2.3.2 Biochemical and Hormonal Assay Methods

2.3.2.1 Estimation of Thyroid Function

2.3.2.1.A Estimation of Serum Thyroid Stimulating Hormone

TSH was measured quantitatively in vitro using an immunoassay. The electrochemiluminescence immunoassay, or "ECLIA," uses two distinct monoclonal antibodies that are targeted

specifically against thyroid-stimulating hormone to generate a sandwich complex and is designed to be used with the Cobas e immunoassay analyzer. The microparticles are magnetically attracted to the surface of the electrode. A photomultiplier measures the chemiluminescent emission that is caused when a voltage is applied to the electrode.

2.3.2.1.B Estimation of Serum Total Thyroxine

The assay of serum total T4 determination is a competitive chemiluminescence immunoassay. First, the sample incubated at 37, thyroxine present in the serum sample competes with the T4 antigen immobilized on the magnetic microbeads for a limited number of binding sites forming immune complexes. A photomultiplier measures the light signal after the chemiluminescent reaction begins, and the light signal is inversely proportional to the amount of T4 in the sample.

2.3.2.1.C Estimation of Serum Free Thyroxine

The assay of serum-free T4 determination is a competitive chemiluminescence immunoassay as explained previously in total T4.

2.3.2.1.D Estimation of Serum Total Triiodothyronine

The assay of serum total T3 determination is a competitive chemiluminescence immunoassay as explained previously in total T4.

2.3.2.1.E Estimation of Serum Free Triiodothyronine

The assay of serum-free T3 determination is a competitive chemiluminescence immunoassay as explained previously in total T4.

2.3.2.1.F Estimation of Serum Diiodothyronine

The approach to the determination of serum diiodothyronine was done by using an enzyme-linked immunosorbant assay (ELISA) kit.

After adding 50 µl of the diluted standard to the standard well and 50 µl of serum to the sample well, then adding 50 µl of biotinylated antigen to each well, the plate was covered with a sealer and incubated for 60 minutes at 37°C. After that, the sealer was removed and the plate was washed five times with 300 µl wash buffer with inverting the plate each time. Then, 50 µl avidin-HRP was added to both the standard well and sample well then the plate was covered with a sealer and incubated for 60 minutes at 37°C. After removing the sealer and washing again, 50 µl substrate solution A was added to each well and then 50 µl of substrate solution B was added to each well. At 37°C, the plate was incubated for 10 minutes. After that, 50 µl stop solution was added to each well and the optical density was determined using a microplate reader set at 450 nm.

2.3.2.1.G Estimation of Serum Reverse Triiodothyronine

This assay was done as the same of diiodothyronine measurment.

2.3.2.2 Determination of Glycemic Indices

2.3.2.2.A Estimation of Fasting Serum Glucose Level

An enzymatic reference technique employing hexokinase, which catalyzes the conversion of glucose to glucose-6-phosphate with the aid of ATP, serves as the foundation for estimate. In the presence of NADP, glucose-6-phosphate dehydrogenase converts glucose-6-phosphate to gluconate-6-phosphate. The carbohydrate is not oxidized in any other way. When measured photometrically with a UV spectrophotometer, the rate of NADPH synthesis during the reaction is perfectly proportional to the glucose concentration.

2.3.2.2.B Estimation of Fasting Serum Insulin Level

Fasting serum insulin level was determined by using an ELISA kit. This kit is based on the sandwich principle. The principle is a two-step incubation method, first, an aliquot of patient samples was incubated in the coated well with enzyme conjugate (which is a monoclonal anti-insulin antibody) that is conjugated with biotin. The biotin anti-insulin antibody is bounded by the streptavidin-peroxidase enzyme complex during the second incubation phase. Inversely correlated with the amount of insulin in the sample is the amount of bound peroxidase complex. With the use of appropriate insulin standards, the color created is proportional to the amount of insulin present in the patient sample. At 450 nm, the absorbance was determined spectrophotometrically. The results were expressed as µIU/ml.

2.3.2.2.C Estimation of Insulin Resistance

Homeostasis model assessment- insulin resistance (HOMA-IR) is a non-invasive, fast, cheap alternative, and reliable way to estimate insulin resistance, was developed by Matthews in 1985 [82], it is calculated from fasting serum insulin and fasting serum glucose using the following formula:

HOMA = FSI (µIU/ml) * FSG (mg/dl) /405

2.3.2.3 Determination of Body Mass Index

A person's weight and height are used to calculate their Body Mass Index (BMI). The body mass index (BMI) is calculated by dividing the body weight by the square of the body height and is represented in kilograms per square meter (kg/m2) with the mass in kilograms and the height in meters.

BMI=Weight / (Height)^2

The values fall in the range between18.5-24.9 represents the normal weight, while the values between 25-30 represents overweight and above 30 is obese [83].

BMI Range	Weight Category
Less than **18.5**	Underweight
Between **18.5** and **24.9**	Normal weight
Between **25** and **29.9**	Overweight
Greater than **30**	Obese

2.3.3 Genetic Analysis

2.3.3.1 Extraction of Genomic DNA from Blood Sample

Favor Prep Genomic DNA Mini kit from Favogen was used to provide a fast and easy method for purification of total DNA from blood and various biological samples and to yield pure DNA suitable for storage and immediate application.

1. A total of 200 µl of whole blood was pipetted and added into 1.5 microcentrifuge tubes.
2. Proteinase K enzyme (30µl) was added to the sample tube and then mix by pulse vortex.
3. FABG buffer (200µl) was added into the sample tube and mixed thoroughly to yield a lysis solution.
4. The mixture was left at room temperature for 2 minutes.
5. The lysate was incubated at 70 ºc for 15 min with repeated mixing.

6. Absolute ethanol (200µl) was added into lysate and mixed well by the vortex.

7. The mixture was applied carefully to the FABG column, close the cap, and centrifuged at 14,000 rpm for 1 min, then the filtrate was discarded and a new collection tube was replaced.

8. A volume of 400µl of W1 buffer was added to the FABG column and centrifuged for 1 min. at 14,000 rpm and discarded the filtrate.

9. A volume of 600µl of Wash buffer was added to the FABG column and centrifuged for 1 min. at 14,000 rpm and discarded the filtrate then the collection tube was replaced with another one for additional centrifuging for 3min. to dry the membrane.

10. The FABG column was replaced with a new 1.5 ml tube then 100 µl of elution buffer was added directly into membrane and incubated for 10min. at room temperature then centrifuged for 1min. at 14,000 rpm to elute the DNA.

2.3.3.2 Conventional Polymerase Chain Reaction

2.3.3.2.A Primer Preparation

Polymerase Chain Reaction (PCR) was performed using a specific primers designed by Dr. Ahmed Abduljabbar to amplify the desired DNA fragments of the DIO2 gene. Two SNPs were investigated, the first is rs205514; 274 T>C SNP and second was rs225017; A>T SNP. Tetra primers amplification refractory mutation system-PCR (tetra ARMS-PCR) technique was used to detect rs205514; 274 T>C SNP and conventional PCR was used to amplify the DNA fragment encompasses rs225017; A>T SNP. Primer-BLAST software was used to create the primers, which were then bought as a lyophilized product from Alpha,

Canada. Each primer was dissolved in specific volumes of nuclease-free water to obtain a stock solution with a concentration of 100 pmol/µl after that, a diluted work solution was made by adding 90µl of nuclease-free water to 10 µl of each stock solution. This work solution was kept at -20°C until use.

Table 2-3: Primers sequences to detect rs225914; 274 T>C SNP and rs225017; A>T SNP

Primers		**Sequence**	**Product size (bp)**
Primers sequences of rs225014 247 T > C	O-F	GAAGTCAGCCACTGAGGAGA ACTC	328 and 216 or 156
	O-R	AATGTGAATTCAAGTGGCAAT GTG	
	I-F	CACTGTTGTCACCTCCTTCGGT	
	I-R	AGTGTGGTGCATGTCTCCATT G	
Primers sequences of rs225017 A > T	F	GGGTGGTTTTTATAGAATTTA GAACA	296
	R	AAGGTACAATCAAAGGATCTT TTGT	

F: Forward primer, R; Reverse primer, OF: Outer forward primer, OR: Outer reverse primer.

2.3.3.2.B Optimization of Polymerase Chain Reaction Conditions

The PCR reaction was optimized following a series of tests to determine the ideal annealing temperature and the ideal number of amplification cycles.

2.3.3.2.C Running the Polymerase Chain Reaction

The PCR reaction was performed by mixing the PCR components with DNA and employing the optimum PCR content as shown in Tables 2-4 and Table 2-5 respectively.

Table 2-4: PCR reaction mixture to amplify a DNA fragment encompasses rs225017; A>T SNP

Component	**Volume (µl)**
Forward primer	2
Reverse primer	2
DNA template	4
Nuclease free water	4.5
Master mix	12.5
Total	25

Table 2-5: PCR reaction mixture to amplify a DNA fragment encompasses rs225014; 274 T>C SNP

Component	**Volume (µl)**
Outer forward primer	1
Outer reverse primer	1

Inner forward primer	1
Inner reverse primer	1
DNA template	4
Nuclease free water	4.5
Master mix	12.5
Total	25

Table 2-6: PCR program to detect rs225914; 274 T>C SNP

Steps	Temperatures/c	Time /second	Cycle
Denaturation	95	5 minutes	1
Initial denaturation	95	40	40
Annealing	59	55	
Extension	72	60	
Final extension	72	5minutes	1

Table 2-7: PCR program to amplify a DNA fragment encompasses rs225017; A>T SNP

Steps	Temperatures/c	Time /second	Cycle
Denature template	95	5 minutes	1
Initial denaturation	95	40	35
Annealing	57	55	
Extension	72	60	

Final extension	72	5 minutes	1

2.3.3.3 Agarose Gel Electrophoresis

1. Agarose gel was prepared by dissolving 1.5g of agarose powder in 100ml of 1x TBE buffer (PH 8).
2. Heating the solution on a hot plate until boiling.
3. While waiting for cooling then 4 µl of ethidium bromide 0.5 µg/mL was added to the solution.
4. The comb was fixed at the end of the tray for making wells used for PCR product loading.
5. The agarose was gently poured into the tray and waited to solidify at room temperature for 30 min.
6. The comb was gently removed from the tray.
7. The tray was fixed in an electrophoresis chamber and filled with a TBE buffer.
8. A DNA ladder was loaded into one well.
9. PCR products were loaded into the wells directly.
10. The electrophoresis apparatus's voltage was set to ensure an electrical field of 5 v for each cm of the distance between the cathode and anode.
11. At the end of the run which is about 90 min, ultraviolet trans-illuminator was used at 320-336 nm for band detection.
12. The gel was photographed using a digital camera.

2.3.3.4 Sequencing and sequence alignment

Sequencing for the amplified products that were obtained to detect rs225017; A>T SNP was done by Macrogen company, Korea

using the forward primer. The National Center for Biotechnology Information (NCBI) website's Basic local alignment search tool (BLAST) and MEGA7 were both used for homology searches to produce the alignment results. The results were compared with reference sequences of the DIO2 gene which obtained from the gene bank as controls.

2.3.4 Statistical Analysis

The data have been analyzed using SPSS version 22 (SPSS Inc, Chicago, USA), a statistical tool for the social sciences. To compare the means of three groups of study subjects (groups obtained regarding rs225014 SNP), one-way analysis of variances (one-way ANOVA) was conducted. A post hoc test after ANOVA test was performed to achieve multiple comparisons between the groups. To compare the means of two groups of the study subjects (groups obtained regarding rs225017 SNP), independent t-test was performed. The Hardy-Weinberg equilibrium's predictions for the distribution of alleles and genotypes were tested using chi-square from goodness of fit test. A significant difference between the means was regarded at $P < 0.05$

3. Results

3.1 Demographic Characteristics of the Hypothyroidism Patients

One hundred fifty hypothyroidism female patients were included in this study, all the patients were from the same ethnicity. The results in Table 3-1 shows that the age mean of the patients was 50.2±11.18 years, the BMI mean was 31.01±6.23 Kg/m^2, and the duration of treatment mean was 4.66±5.41 years.

Table 3-1: The demographic characteristics of the hypothyroidism patients

Parameters	**Mean ±SD (N= 150)**	**Minimum**	**Maximum**
Age (years)	50.20± 11.18	40.00	74.00
BMI (Kg/m^2)	31.01±6.23	15.37	48.52
Duration of the treatment (years)	4.66±5.41	0.33	21.00

N: Numbers of the study subjects, SD: Standard deviation, BMI: Body mass index

A loss of 20 DNA samples occurred due to the continuous work repetition to reach the optimized PCR conditions, accordingly DNA samples for only 130 patients were genotyped regarding rs225017 SNP. The results in Table 3-2 show the demographic characteristics of 130 patients who were genotyped for rs225017 SNP. The age mean was 48.74± 8.79 years, the BMI mean was 29.99± 5.76 Kg/m2, and the duration of treatment mean was 3.95± 3.58 years.

Table 3-2: The demographic characteristics of the hypothyroidism patients who was genotyped for rs225017 SNP

Parameters	Mean ±SD (N= 130)	Minimum	Maximum
Age (year)	48.74± 8.79	40.00	74.00
BMI (Kg/m^2)	29.99± 5.76	15.38	45.92
Duration of the treatment (years)	3.95± 3.58	0.33	17.00

N: Numbers of the study subjects, SD: Standard deviation, BMI: Body mass index.

3.2 The Biochemical Characteristics of the Hypothyroidism Patients

The biochemical analysis that has been performed for 150 patients included the TSH, the thyroid hormones and some glycemic indices (fasting blood sugar, fasting serum insulin and HOMA-IR). The results revealed that there was a large difference in the TSH among the hypothyroidism patients, while the other thyroid hormones were normally distributed. Regarding the glycemic indices, insulin and HOMA-IR were largely different among the patients while the other fasting blood sugar was normally distributed (Table 3-3).

Table 3-3: The biochemical parameters of the hypothyroidism patients

Parameter	Mean± SD (N =150)	Minimum	Maximum	Normal Values
TSH (µIU/mL)	6.03±10.71	0.005	100.00	0.4-5
Total T3 (nmol/L)	1.54±0.46	0.01	3.61	0.92-2.33
Total T4 (nmol/L)	103.04±30.34	54.50	390.30	64.3-185
Free T3 (pmol/L)	6.53±1.55	3.20	14.60	2-9
Free T4 (pmol/L)	15.27±4.07	3.10	40.30	9-20
rT3 (pmol/L)	925.31±373.49	377.57	2135.55	
T2 (pmol/L)	2076.15±1191.13	157.67	5493.03	
T3/T4	1.55±0.42	0.001	2.75	
FT3/FT4	0.44±0.12	0.23	1.03	
T3/rT3	5.23±1.89	0.001	5.23	
rT3/T4	0.95±0.43	0.24	2.39	
FSI (mIU/mL)	18.08±18.16	2.83	100.00	4-23.4
FBG (mg/dL)	111.20±47.02	70.00	432.00	70-120
HOMA-IR	4.88±4.80	0.61	23.73	

T4 dose (mcg)	90.33±37.25	10.00	200.00	

N: Numbers of the study subjects, SD: Standard deviation, T3: 3,3,5-Triiodothyronine, T4: Thyroxin, T2: 3,5- Diiodothyronine, rT3: reverse triiodothyronine, FBS: Fasting blood sugar, FSI: Fasting serum insulin, HOMA-IR: Homeostatic model assessment for insulin resistance

The results in Table 3-4 shows the biochemical parameters of 130 patients with rs225017 SNP.

Table 3-4: The biochemical parameters of the hypothyroid patients with rs225517 SNP

Parameter	Mean± SD (N =130)	Minimum	Maximum	Normal Values
TSH (µIU/mL)	5.73±7.65	0.01	40.00	0.4-5
Total T3 (nmol/L)	1.54±0.48	0.01	3.61	0.92-2.33
Total T4 (nmol/L)	103.46±30.77	58.50	29.30	64.3-185
Free T3 (pmol/L)	6.45±154	3.90	14.60	2-9
Free T4 (pmol/L)	15.39±4.09	2.20	40.30	9-20
rT3 (pmol/L)	918.57±363.58	377.57	2135.55	
T2 (pmol/L)	2048.23±1179.69	157.67	5493.02	
T3/T4	1.55±0.42	0.83	2.75	

FT3/FT4	0.43±0.11	0.23	0.81	
T3/rT3	1.91±0.82	0.66	5.23	
rT3/T4	0.94±0.43	0.24	2.26	
Insulin mIU/mL	18.63±18.86	3.61	100	4-23.4
FBG mg/dL	111.06±48.77	70.00	432.00	70-120
HOMA-IR	4.95±4.78	0.81	23.73	
T4 dose (mcg)	88.34±36.54	10.00	200.00	

N: Numbers of the study subjects, SD: Standard deviation, T3: 3,3,5 Triiodothyronine, T4: Thyroxin, T2: 3,5- Diiodothyronine FBS: Fasting blood glucose, rT3: reverse triiodothyronine, HOMA-IR: Homeostatic model assessment for insulin resistance

3.3 The Blood Pressure Parameters of the Hypothyroidism Patients

Table 3-5 illustrates the blood pressure parameters of the study subjects. The results showed that the mean of the systolic and diastolic BP were within the normal ranges regarding the age of the patients. The mean of arterial pressure of the patients were within the normal range as well.

Table 3-5: The blood pressure parameters of the hypothyroidism patients of rs225014

Parameter	Mean± SD (N =150)	Minimum	Maximum
Systolic BP (mmHg)	125.83±15.16	100.00	180.00
Diastolic BP (mmHg)	81.61±6.98	60.00	110.00
MAP (mmHg)	96.35±9.09	73.33	126.67

Bp: Blood pressure, MAP: Mean arterial pressure.

The results in table 3-6 shows the blood pressure parameters of 130 patients with rs225017.

Table 3-6: Blood pressure parameters of the hypothyroid patients who was genotyped for rs225017 SNP

Parameter	Mean± SD (N =130)	Minimum	Maximum
Systolic BP (mmHg)	124.61±13.92	80	180
Diastolic BP (mmHg)	81.46±6.60	60	110
MAP (mmHg)	96.35±9.09	73.33	126.67

Bp: Blood pressure, MAP: Mean arterial pressure.

3.4 The Genetic Analysis

3.4.1 Analysis of rs225014; 274 T>C SNP

After performing tetra ARMS- PCR, the detection of rs225014; 274 T>C SNP was done by performing horizontal agarose gel electrophoresis. This technique uses 4 primers, 2 of them are outer primers and the other 2 are inner primers. The inner forward primers are designed to work when there is no SNP and give a band appears in the agarose gel at the size of 156 bp and the inner reverse primer is designed to work when the SNP is present and give a band appears in the agarose gel at the size of 216 bp. The outer primers work anyway and give a

'control' band with the size 328 bp. Thus, the sizes of the PCR products vary according to the presence or absence of the SNP. Two DNA bands of the sizes 328 bp and 156 bp were obtained in the case of the wild type (TT), while three DNA bands of the sizes 328 bp, 216 bp and 156 bp were obtained in the case of the heterozygous mutant type (TC), and two DNA bands of the sizes 328 bp and 216 bp were obtained in the case of the homozygous mutant type (CC) (Figure 3-1).

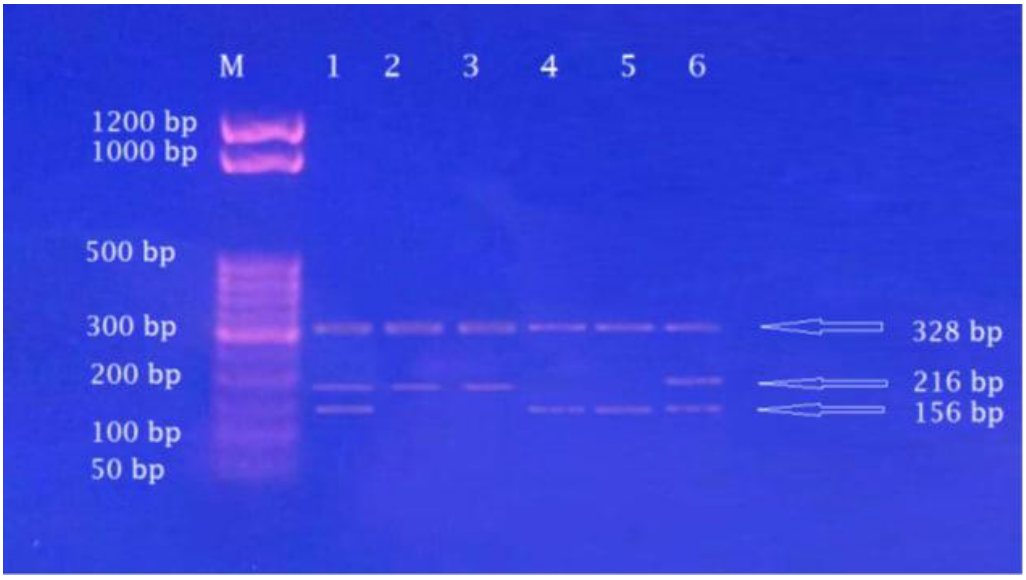


Figure 3-1: The horizontal agarose gel electrophoresis (1.5% w/v) of amplification refractory mutation system-polymerase chain reaction detecting rs225014; 274 T>C SNP. M: 50 bp DNA marker. Lane 1 and 6 shows the heterozygous mutant type (TC). Lane 2 and 3 shows the homozygous mutant type (CC). Lane 4 and 5 shows the wild type (TT).

The distribution of the alleles and genotypes of this SNP is illustrated in Table 3-7. The distribution revealed the prevalence of the C allele in the study subjects.

Table 3-7: The distribution of alleles and genotypes of rs225014; 274 T>C in the study subjects

Genotype (N=150)	Frequency (%)	Allele	Frequency	Chi-square	P-value
TT (Wild type)	22 (14.7)	T	0.21	49.12	0.0001
TC (Heterozygous mutant type)	19 (12.7)	C	0.79		
CC (Homozygous mutant type)	109 (72.7)				

N: Numbers of the study subjects

3.4.2 Analysis of rs225017; A>T SNP

The amplified DNA fragment was of the size 296 bp as shown in Figure 3-2.

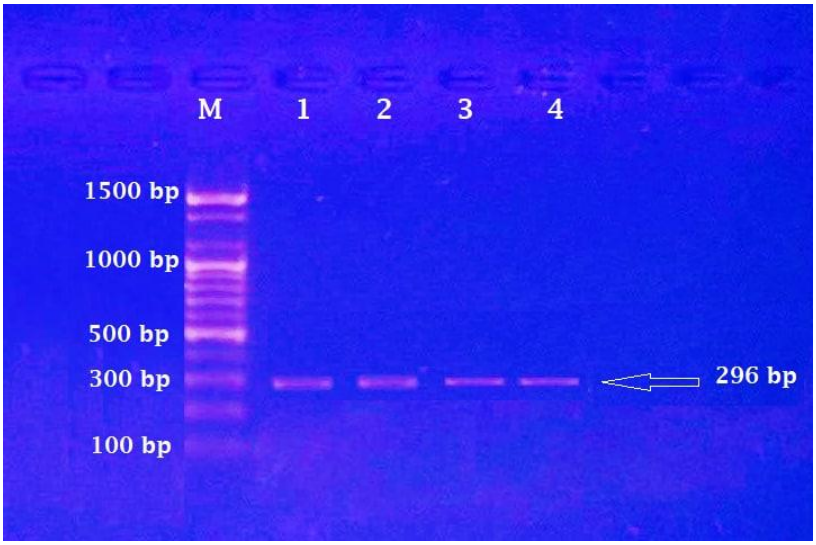


Figure 3-2: The horizontal agarose gel electrophoresis (1.5% w/v) of conventional PCR detecting rs225017; A>T. M: 100 b.p DNA ladder.

The sequence of the PCR product regarding rs225017; A>T SNP was analyzed by alignment to the Homo sapiens DIO2, transcript variant 1, mRNA reference sequence using the basic local alignment search tool (BLAST) on the national center for biotechnology information (NCBI) web site (Figure 3-3).

Homo sapiens iodothyronine deiodinase 2 (DIO2), transcript variant 1, mRNA

Sequence ID: **NM_013989.5, Number of Matches: 1**

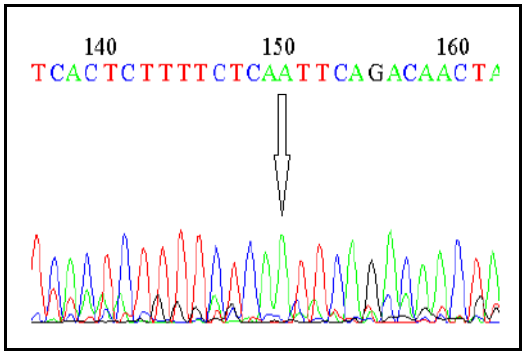
Score	Expect	Identities	Gaps	Strand
442 bits(239)	0,0	241/242(99%)	0/242(0%)	Plus/Plus

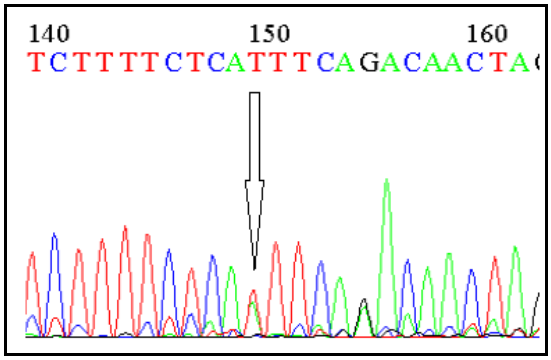
```
Query  1     CAGTCCCAATGGAGCTTTAATATTTACACAATATACCAGCTGGTGACACGACTGATATGT  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2788  CAGTCCCAATGGAGCTTTAATATTTACACAATATACCAGCTGGTGACACGACTGATATGT  2729

Query  61    TGCTCCCATTTAAAAAATAAAATAAAATACGTTTGGTTCTTGCACACCTAGTTCTCACTC  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2728  TGCTCCCATTTAAAAAATAAAATAAAATACGTTTGGTTCTTGCACACCTAGTTCTCACTC  2669

Query  121   TTTTCTCATTCAGACAACTAGTTTAAGAATGTATTTTCAACTTTAGTTTTTTTCTATCC  180
             |||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2668  TTTTCTCAATTCAGACAACTAGTTTAAGAATGTATTTTCAACTTTAGTTTTTTTCTATCC  2609

Query  181   ATTTTATTTATTTTTTATTCAGTTATTAATGGAATGGACAAAAGATCCTTTGATTGTACC  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2608  ATTTTATTTATTTTTTATTCAGTTATTAATGGAATGGACAAAAGATCCTTTGATTGTACC  2549

Query  241   TT  242
             ||
Sbjct  2548  TT  2547
```

Figure 3-3: Alignment using BLAST on NCBI shows rs225017; A>T in DIO2 gene. Query represents the sample sequence, Sbject represents the sequence of the database of NCBI. The bit Score is statistical measure of the moral similarity. Expect is an estimation of the number of times expected to get the same similarity.

Three models of genotypes regarding rs225017; A>T SNP were detected after PCR products sequence analysis, they were: AA (the wild type), AT (the heterozygous mutant type and TT (the homozygous mutant type) as illustrated in Figure 3-4.

A **B**





C

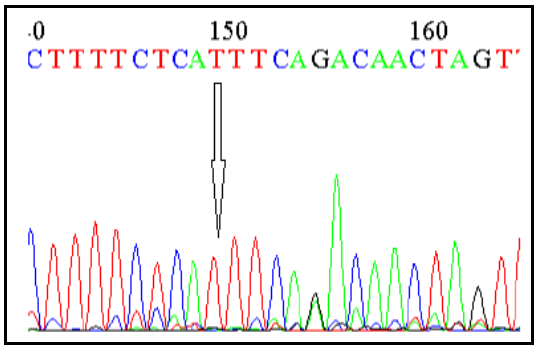


Figure 3-4: Direct sequencing of forward strand to detect rs225017; A>T SNP in DIO2 gene. A: Wild type pattern (AA). B: Heterozygous mutant type (AT). C: Homozygous mutant type (TT).

Figure 3-4 shows parts of the chromatograms of the direct sequencing of three independent samples. Part A shows the wild type pattern with the green peak indicated by the arrows representing adenine. Part B shows the heterozygous mutant type with two overlapping peaks indicated by the arrows, one with red color represents thymine and the other with green color represents adenine. Part C shows the homozygous mutant type with one red peak indicated by the arrows represents thymine.

The distribution of the alleles and genotypes of this SNP was illustrated in Table 3-8. The distribution revealed the prevalence of the A allele in the study subjects. There was one patient who carried the TT genotype among the study population.

Table 3-8: The distribution of alleles and genotypes of rs225017; A>T in the study subjects

Genotype (N=130)	Frequency (%)	Allele	Frequency	Chi-square	P-value
AA (Wild type)	120 (92.3)	A	0.957	2.78	0.24
AT (Heterozygous mutant type)	9 (6.9)	T	0.042		
TT (Homozygous mutant type)	1 (0.8)				

N: Numbers of the study subjects

3.5 The Association between the DIO2 polymorphisms and the Demographic Characteristics

The analysis of the association between the rs225014; 274 T>C SNP and the demographic characteristics showed no significant difference between the three groups regarding age, BMI, and duration of treatment, as shown in Table 3-9.

Table 3-9: The demographic characteristic according to the genotypes of rs225014; 274 T>C SNP among the hypothyroidism patients

Demographic characteristic	Patients' genotypes (N= 150)			P-value
	TT (N=22)	TC ((N=19)	CC (N=109)	
Age (year)	50.19±2.80	51.63±2.80	49.96±1.06	0.83
BMI (Kg/m^2)	32.44±1.54	30.80±1.12	30.770.60	0.53
Duration of the treatment (years)	4.96±0.69	5.26±0.86	3.99±0.38	0.28

The data represented as mean± standard error of the mean, N: Numbers of the study subjects.

As with rs225014; 274 T>C SNP, the analysis of the association between the rs225017; A>T SNP and the demographic characteristics showed no significant difference between the three groups regarding age, BMI, and duration of treatment, as shown in Table 3-10.

Table 3-10: The demographic characteristic according to the genotypes of rs225017; A>T SNP among the hypothyroidism patients

Demographic characteristic	Patients' genotypes (N= 130)			P-value
	AA (N=120)	AT (N=9)	TT (N=1)	
Age (year)	48.59±0.79	51.77±10.09	40	0.354
BMI (Kg/m^2)	29.99±0.51	29.05±2.31	38.39	0.31
Duration of the treatment (years)	4.12±0.33	2.05±0.70	1.00	0.17

The data represented as mean± standard error mean, N: Numbers of the study subjects.

3.6 The Association between the DIO2 polymorphisms and the Thyroid Hormones

This study demonstrated that according to rs225014; 274 T>C genotypes groups, there were no significant differences of the total T3, free T3, free T4, TSH, rT3, T2, and levothyroxine dose, but there was a significant difference in total T4; the TC genotype group has a higher serum value than CC genotype group. As a consequence for the difference in the total T4, T3/T4 molar ratio was significantly different between the groups (Table 3-11).

Table 3-11: The thyroid hormones according to the genotypes of rs225014; 274 T>C SNP among the hypothyroidism patients

Parameter	Patients' genotypes (N= 150)			P-value
	TT (N=22)	TC ((N=19)	CC (N=109)	
Total T3 (nmol/L)	1.70±0.14	1.49±0.06	1.52±0.04	0.235
Free T3 (pmol/L)	7.05±0.30	6.04±0.25	6.51±0.15	0.118
Total T4 (nmol/L)	112±8.07	115±3.05	99.04±2.90	0.027* c
Free T4 (pmol/L)	16.64±1.14	15.23±0.67	15.02±0.38	0.248
TSH (µIU/mL)	5.70±1.73	5.07±1.07	6.26±1.13	0.896
rT3 (pmol/L)	937.38±90.39	911.46±78.72	925.40±35.80	0.977
T2 (pmol/L)	2035.72±213.92	2151.36±233.47	2070.83±120.86	0.951
T3/T4	1.54±0.10	1.32±0.08	1.59±0.03	0.043* c
FT3/FT4	0.45±0.03	0.40±0.06	0.45±0.12	0.27
T3/rT3	2.03±0.18	1.82±0.14	1.89±0.07	0.27
rT3/T4	0.87±0.08	0.80±0.07	0.99±0.04	0.14
T4 dose (µg)	91.66±10.10	85.52±7.23	90.91±3.49	0.833
Post hoc test: c: TC vs CC				

The data represented as mean± standard error of the mean, N: Numbers of the study subjects, T3: 3,3,5 Triiodothyronine, T4: Thyroxin, T2: 3,5- Diiodothyronine FBS: Fasting blood glucose, rT3: reverse Triiodothyronine.

Regarding rs225017; A>T SNP, the results showed that there was no association between various genotypes and levothyroxine dose, total T3, total T4, free T3, free T4, TSH, and free T3 levels. Meanwhile , the

results showed that there was a significant difference in the T2 level and T3/T4 molar ratio among the three groups as shown in Table 3-12.

Table 3-12: The thyroid hormones according to the genotypes of rs225017; A>T SNP among the hypothyroid patients

Parameter	Patients' genotypes (N= 130)			P-value
	AA (N=120)	AT (N=9)	TT (N=1)	
Total T3 (nmol/L)	1.54±0.04	1.50±0.07	1.95	0.79
Total T4 (nmol/L)	104.82±2.87	88.74±3.84	72.70	0.13
Free T3 (pmol/L)	6.45±0.14	6.25±0.25	8.20	0.72
Free T4 (pmol/L)	15.39±0.38	15.08±0.62	18.20	0.82
TSH (µIU/mL)	5.76±0.71	5.62±2.22	3.12	0.95
rT3 (pmol/L)	920.65±32.38	810.15±141.56	1643.86	0.37
T2 (pmol/L)	2123.41±108.78	1149.61±154.62	1114.75	0.016*
T3/T4	1.53±0.03	1.70±0.07	2.68	0.014*
FT3/FT4	0.43±0.01	0.42±0.02	0.45	0.77
T3/rT3	1.89±0.07	2.25±0.34	1.18	0.25
rT3/T4	0.93±0.03	0.93±0.17	2.26	0.97
T4 dose (µg)	88.95±3.37	78.88±11.23	100.00	0.41

The data represented as mean± standard error of the mean, N: Numbers of the study subjects, T3: 3,3,5-Triiodothyronine, T4: Thyroxin, T2: 3,5- Diiodothyronine, rT3: reverse Triiodothyronine, *: significant at P≤0.05.

3.7 The Association between the DIO2 polymorphisms and the Glycemic Profile

Statistical analysis was conducted to reveal the association between the rs225014; 274 T>C SNP and the glycemic profile, the results indicated a significant difference both in fasting insulin and HOMA-IR; the CC genotype group has lower fasting insulin and HOMA-IR than the

TC genotype group, but there was no significant difference regarding fasting blood sugar (Table 3-13).

Table 3-13: The glycemic parameters according to the genotypes of rs225014; 274 T>C SNP among the hypothyroidism patients

Parameter	Patients' genotypes (N= 150)			P-value
	TT (N=22)	TC (N=19)	CC (109)	
FBS mg/dL	106.68±7.90	119.55±10.07	110.62±4.73	0.670
Fasting insulin mIU/mL	19.44±4.57	29.51±6.96	15.83±1.31	0.009 [c]
HOMA-IR	5.22±1.23	7.88±1.55	4.29±0.38	0.010 [c]
Post hoc test: c: TC vs CC				

The data represented as mean± standard error of the mean, N: Numbers of the study subjects, FBS: Fasting blood sugar, FSI: Fasting serum insulin, HOMA-IR: Homeostatic model assessment for insulin resistance.

The results indicated that there were no significant differences among the three groups of patients regarding rs225017; A>T SNP in fasting blood sugar, fasting serum insulin, and HOMA-IR values, as shown in Table 3-14.

Table 3-14: The glycemic parameters according to the genotypes of rs225017; A>T SNP among the hypothyroid patients

Parameter	Patients' genotypes (N= 130)			P-value
	AA (N=120)	AT (N=9)	TT (N=1)	
FBS mg/dL	18.69±1.75	18.93±5.01	8.88	0.87
Fasting insulin mIU/mL	112.64±4.59	91.41±3.40	99.00	0.44
HOMA-IR	5.04±0.44	4.13±0.92	2.17	0.72

The data represented as mean± standard error of the mean, N: Numbers of the study subjects, FBS: Fasting blood sugar, FSI: Fasting serum insulin, HOMA-IR: Homeostatic model assessment for insulin resistance.

3.8 The Association between DIO2 polymorphisms and Blood Pressure Parameters

The association between rs225014; 274 T>C SNP and the blood pressure parameters was analyzed and the results indicated that there were no significant differences among all the three groups of the patients regarding systolic, diastolic, and mean arterial pressure (Table 3-15).

Table 3-15: The blood pressure parameters according to the genotypes of rs225014; 274 T>C SNP among the hypothyroidism patients

Parameter	Patients' genotypes (N= 150)			P-value
	TT (N=22)	TC ((N=19)	CC (N=109)	
Systolic BP (mmHg)	128.09±3.20	127.36±3.57	125.13±1.46	0.643
Diastolic BP (mmHg)	83.33±1.59	81.57±1.38	81.28±0.67	0.471
MAP (mmHg)	98.25±1.86	96.84±1.98	95.90±0.89	0.541

The data represented as mean± standard error mean, N: Numbers of the study subjects, BP: Blood pressure, MAP: Mean arterial pressure.

The results indicated that there was no significant association between the rs225017; A>T SNP and blood pressure parameters represented by systolic, diastolic, and mean arterial pressure (Table 3-16).

Table 3-16: The blood pressure parameters according to the genotypes of rs225017; A>T SNP among the hypothyroidism patients

Parameter	Patients' genotypes (N= 130)			P-value
	AA (N=120)	AT (N=9)	TT (N=1)	
Systolic BP (mmHg)	124.66±1.30	124.44±3.37	120.00	0.94
Diastolic BP (mmHg)	81.50±0.62	81.11±111	80.00	0.96
MAP (mmHg)	95.88±0.78	95.55±1.84	93.33	0.95

The data represented as mean± standard error mean, N: Numbers of the study subjects, BP: Blood pressure, MAP: Mean arterial pressure.

3.9 The Correlation between the DIO2 Genetic Polymorphisms and the Significantly Different Parameters

Eventually, Total T4, serum insulin, HOMA-IR, and T3/T4 ratio showed a significant difference between the genotypes of the rs225014 SNP. To further analyze the effect of the rs225014 SNP on these parameters, the estimated effect size represented by Eta and partial eta quared was calculated (Table 3-17).

Table 3-17: The correlation between rs225014; 274 T>C SNP and some of the biochemical parameters

Parameters	Eta	Partial Eta Squared	P-value
Total T4	0.219	0.048	0.027
T3/T4	0.206	0.042	0.043
Fasting insulin	0.251	0.063	0.009
HOMA-IR	0.284	0.062	0.010

Eta represents the estimated effect size of the independent variable.

Figure 3-5, Figure 3-6, Figure 3-7 and Figure 3-8 indicted the estimated marginal means of total T4, T3/T4 molar ratio, fasting serum insulin and HOMA-IR, respectively distributed on the three genotypes groups of rs225014; 274 T>C.

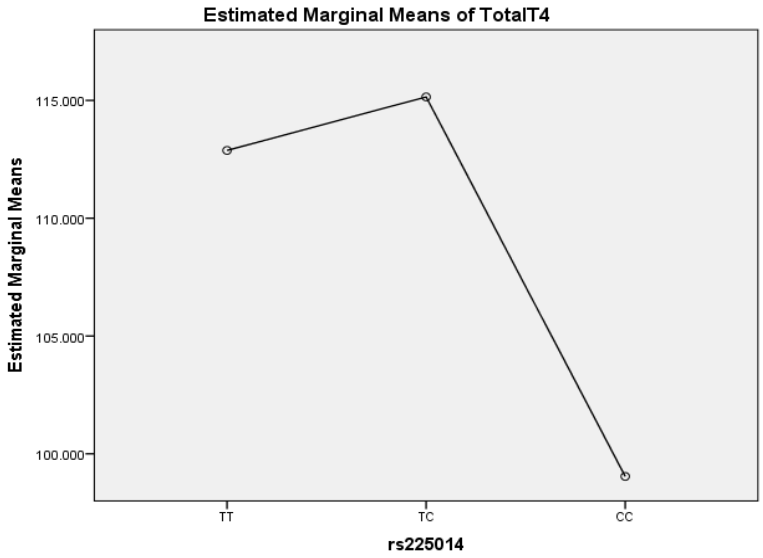


Figure3-5: The correlation between rs225014; 274 T>C and total T4

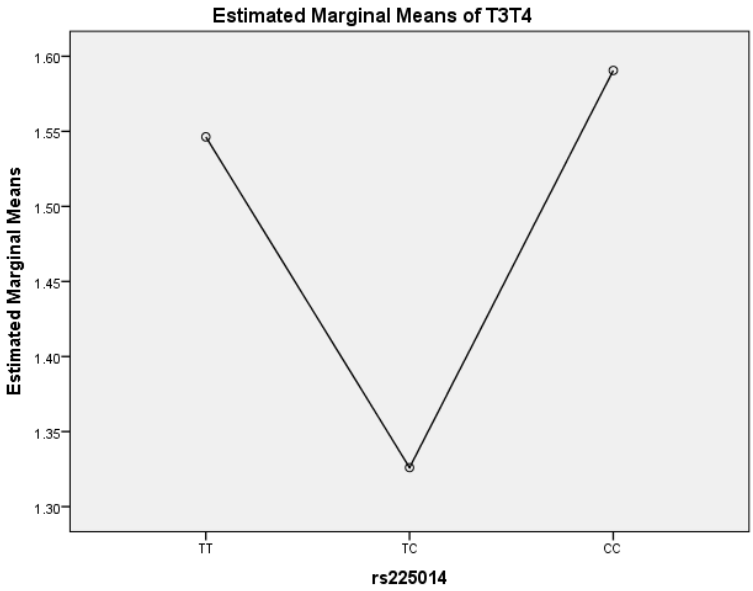


Figure3-6: The correlation between rs225014; 274 T>C and total T3/T4

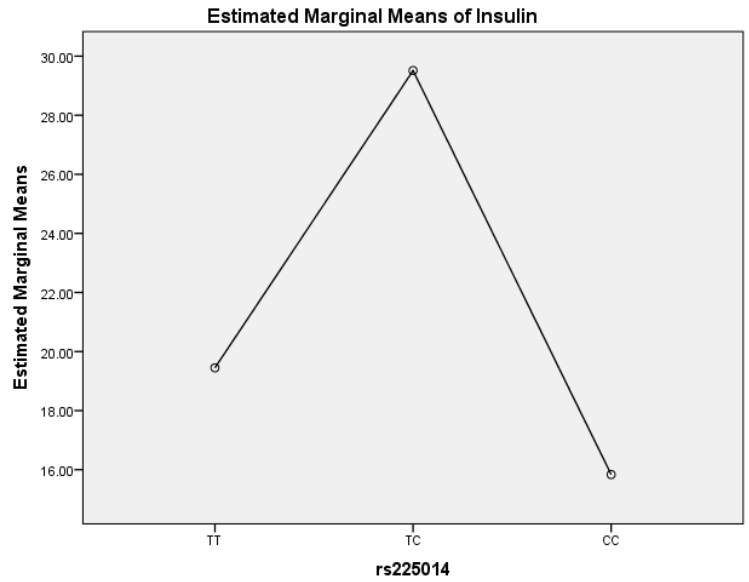


Figure3-7: Correlation between rs225014; 274 T>C and total fasting serum insulin

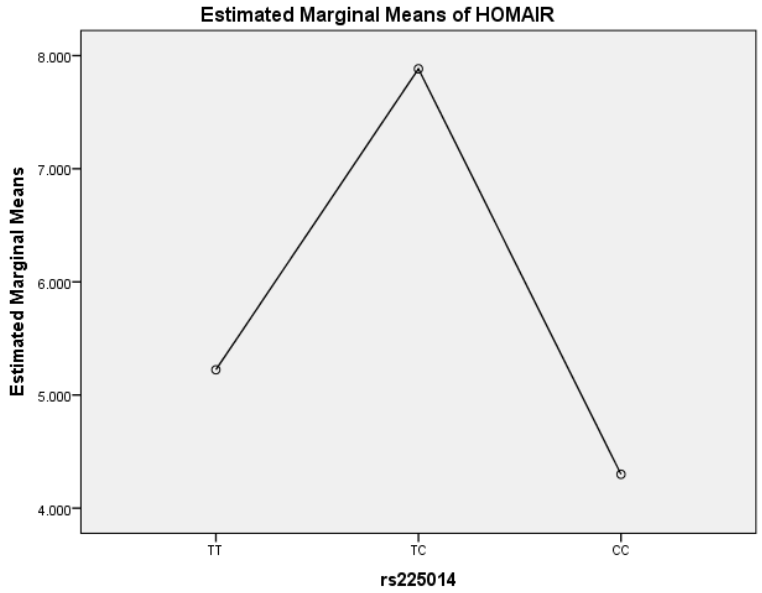


Figure3-8: The correlation between rs225014; 274 T>C and total HOMA-IR

The data for the T2 level and T3/T4 ratio showed a significant difference between the alleles of the rs225017 SNP. To further analyze the effect of the rs225017 SNP on these parameters, the estimated effect size represented by Eta and Partial Eta Squared was calculated (Table 3-18).

Table3-18: The correlation between rs225017; A>T SNP and some of the biochemical parameters

Parameters	Eta	Partial Eta Squared	P-value
T2	0.211	0.044	0.016
T3/T4	0.100	0.010	0.257

Eta represents the estimated effect size of the independent variable.

3.10 The Impact of the Presence of both rs225014 SNP and rs225017 SNP on the Study Parameters

The results in Table 3-19 demonstrate the effect of both the two SNPs on the different estimated parameters and characteristics in the hypothyroid patients. No statistically significant difference ($p > 0.05$) was found in all parameters (demographics characteristics, thyroid hormones, blood pressure and glycemic parameters).

Table3-19: The Impact of the Presence of both rs225014 SNP and rs225017 SNP on the Study Parameters

Parameter	Patients' genotypes (N= 130)			P-value
	No SNP (N= 18)	Either rs225014 or rs225017 SNP (N= 103)	Both SNPs (N= 9)	
Age	47.88±2.24	49.33±0.95	56.33±4.28	0.09
BMI	32.96±1.72	30.26±0.59	31.69±2.10	0.22
Duration of treatment	4.90±0.73	3.84±0.34	2.12±0.68	0.13
Total T3 (nmol/L)	1.73±0.17	1.51±0.43	1.55±0.29	0.18

Free T3 (pmol/L)	6.99±0.35	6.35±0.15	6.50±0.46	0.27
Total T4 (nmol/L)	114.24±9.36	103±2.91	103.92±11.82	0.38
Free T4 (pmol/L)	16.64±1.32	15.17±3.91	15.56±1.19	0.38
TSH (µIU/mL)	6.28±1.99	5.64±0.75	2.14±0.58	0.36
rT3 (pmol/L)	990.26±99.92	904.33±33.79	741.79±109.69	0.23
T2 (pmol/L)	2037.06±237.32	2130.89±120.35	1180.66±154.70	0.06
T3/T4	1.56± 0.11	1.52±0.04	1.56±0.11	0.90
FT3/FT4	0.45±0.03	0.43±0.01	0.43±0.04	0.74
T3/rT3	1.95±0.20	1.87±0.08	2.49±0.41	0.12
rT3/T4	0.91±0.09	0.93±0.04	0.77±0.12	0.54
T4 dose (µg)	88.88±11.29	88.59±3.44	94.44±10.01	0.90
FBS mg/dL	109.46±9.08	112.95±5.13	96.52±8.32	0.62
Fasting insulin mIU/mL	20.75±5.26	18.33±1.83	17.92±5.91	0.87
HOMA-IR	5.66±1.41	4.92±0.46	4.16±1.07	0.72
Systolic BP (mmHg)	129.44±3.65	124.85±1.37	118.88±2.60	0.17
Diastolic BP (mmHg)	83.88±1.83	81.06±0.64	77.77±2.22	0.07
MAP (mmHg)	99.07±2.11	95.66±0.83	91.48±2.29	0.08

4. Discussion

Hypothyroidism is a common and chronic endocrine disease caused by underproduction of thyroid hormones leading to alteration in many aspects like growth and metabolism.

Levothyroxine is the synthetic form that has the same action and structure of the natural endogenous thyroid hormone. Levothyroxine act to neutralize TSH value and correct the symptoms of thyroid deficiency, therefore it is indicated in primary, secondary, and tertiary hypothyroidism [(10)].

Deiodinase type 2 is one of the deiodinases family, its function is to convert T4 to T3 by deiodination at the phenolic ring (outer ring deiodination) [(65)]. It is a single-copy gene that is located on the long arm of the 14th human chromosome at location 14q24.3. Its coding sequence is separated into two exons by an approximately 7.4-kb intron [(69)].

The ultimate goal of personalized medicine is to perfectly match each treatment action with the molecular profile of the patient. The development of sequencing technologies has accelerated the study of human genetics during the past twenty years, providing a better knowledge of the association between genetic variation and human health. Personalized medicine has made extensive use of genetics, and one of its more recent uses is pharmacogenomics-informed pharmacotherapy, which adjusts drug choice and dosage based on a patient's genetic characteristics [(84)].

There is a clinical observation in the local population that hypothyroid patients on hormonal replacement therapy still suffer from disease-related symptoms. Not all treated patients have standard quality of life values [(85)].

Since genetic differences are one of the most determinants of drug response, it was a rationale to see the extent of these polymorphisms on levothyroxine response.

This study is the first of its kind to investigate the effect of DIO2 gene polymorphisms; rs225014; Thr92Ala; 274 T>C and rs225017; A>T in the Iraqi female hypothyroid patients.

4.1. Demographic Characteristics of the hypothyroid Patients

Hypothyroidism incidence increases with age and the value of BMI, it is also more common in females than males and in white people than other races [11].

It was reported that the genetic polymorphisms in the deiodinase type 2 enzyme affect its catalytic ability, it is hypothesized that those polymorphisms can affect the metabolic parameters and may raise the BMI values of the SNPs carriers [75].

This study showed that rs225014 SNP does not affect BMI values (Table 3-9). This result comes along with Nair and colleagues who failed to find a correlation between this polymorphism and BMI values in Pima Indians community in USA [86]. However, a very recent meta-analysis global study by Wang and colleagues found that the DIO2 Thr92Ala polymorphism was associated with a higher BMI [87]. An explanation for this, is that most of our sample of patients were post-menopausal women and since postmenopausal women have higher BMI values, this led to a homogenous sample with no significant difference.

Our results showed no association between rs225017 SNP and BMI values (Table 3-10), and that comes along with what Leiria and

colleagues who have found the same results in their study in Brazil population [81].

4.2 The Association between DIO2 Genetic Polymorphisms and Thyroid Hormones

Because of the finding that both the serum and intracellular T3 levels rely mostly on the peripheral conversion of T4 to T3 mediated by the DIO2 enzyme, and since DIO2- knockout mice have abnormal thyroid parameters [88], the hypothesis was that any disruption to DIO2 gene such as genetic polymorphism may lead to altered thyroid parameters.

4.2.1 The Association between the rs225014; 274 T>C SNP and Thyroid Parameters

As demonstrated in Table 3-11; this study showed no association between rs225014; Thr92Ala; 274 T>C and TSH value.

This finding comes along with a large cohort study of 12,625 participants from western Europe decency done by Wouters and colleagues, who found that there were no significant associations between this SNP and the TSH in the levothyroxine users as well as in the general population [79]. Heemstra and colleagues also found that in a study of the Dutch population, no variations in the relationship between TSH level for various carriers of the rs225014; Thr92Ala SNP in patients treated for Hashimoto thyroiditis were revealed [80]. Butler and colleagues also mentioned that no significant differences were noted in baseline serum TSH among the three genotype groups in a study conducted in Maryland, USA [75].

While Arici and colleagues revealed that there was a statistically significant relationship between the rs225014 SNP and TSH levels; a study on the Turkish population indicated that homozygous wild type (TT) was linked to higher levels of TSH (p 0.05) [89].

As shown in Table 3-11, this study showed no association between rs225014; Thr92Ala; 274 T>C SNP and the thyroid parameters except for total T4 where; the TC carriers group has a higher value than the CC group.

This finding comes along with Wouters and colleagues cohort study on Western Europe decency patients, they studied thyroid hormone parameters among the three genotypes carriers of rs225014 SNP, the total study population was 12,625 participants. No statistically significant differences were observed in free T4, free T3 and freeT3/free T4 values [79]. In their study on the Dutch population, Heemstra and colleagues also stated that there were no differences found in the free T4, total T3, and rT3 values between wild-type, heterozygous, and homozygous carriers of the rs225014 polymorphism in patients treated for Hashimoto thyroiditis [80] Butler and colleagues also mentioned that no significant differences were noted in the baseline serum free T4 and total T3 levels by genotype group groups in a study conducted in Maryland, USA [75].

While Castanga and colleagues in his study conducted on the Italian population found an association between low free T3 values and rs225014 polymorphism; specifically, the mean free T3 levels were significantly lower in patients carrying the mutant allele than in wild-type patients [76].

The lack of the significant association between rs225014; Thr92Ala; 274 T>C SNP and thyroid hormones except total T4 could due

to the fact that there are other genes that control the metabolism and regulation of these hormones such as DIO1, DIO3, TSH receptor gene, or even thyroid transporters. The polymorphisms in these genes could be the main cause of the reduced response to levothyroxine. At the same time DIO2 and rs225014 SNP cannot be ruled out from being a cause of hypothyroidism or for the lack of response to levothyroxine therapy because the homozygous mutant type (CC) of this SNP was the most frequent genotype in our sample of Iraqi hypothyroidism patients.

As shown in Table 3-11; this study showed no linkage between rs225014; Thr92Ala; 274 T>C and levothyroxine dose.

This result comes along with what Santoro *et al* have found in the Brazilian population [(90)]. Heemstra and colleagues also demonstrated that no differences were observed in levothyroxine dose between wild-type, heterozygous and homozygous carriers of this polymorphism in patients treated for Hashimoto thyroiditis [(80)].

While in a study on the Italian population, Torlontano and colleagues reported that patients homozygous for the mutant allele required a greater levothyroxine dose when compared to heterozygous and wild type patients in order to meet their target TSH level [(74)].

4.2.2 The Association between the rs225017; A>T SNP and Thyroid Parameters

The deiodinase type 2 enzyme catalyzes the removal of the outer-ring iodine from thyroxine (T4) to form bioactive T3, which is what determines T3 availability at the cellular level. T2 is the result of thyroid hormone metabolism, therefore converting T3 to 3,5-T2 would need a comparable outer-ring deiodination [(91)].

This study revealed that there was no association between rs225017 SNP and thyroid hormones parameters except for T2, T2/T4 molar ratio, and rT3/T4 molar ratio as demonstrated in Table 3-12. There were no available previous studies on rs225017 regarding thyroid hormones parameters to compare with.

4.3 The Association between the DIO2 Genetic Polymorphisms and the Glycemic Profile

The DIO2 enzyme has an essential role in multiple metabolic pathways, accordingly it hypothesized that the genetic polymorphisms in this gene may cause a change in the glycemic profile of SNP-carrier patients.

This study showed that rs225014 affects serum insulin and HOMA-IR values in our sample of the patients (Table 3-13), the heterozygous group (TC) has higher values of both serum insulin and HOMA-IR than the homozygous group (CC). While there was no relationship between this SNP and fasting blood glucose.

In previous study, Zhang and colleagues in their global meta-analysis found that an individuals with type 2 diabetes mellitus, homozygosity for the DIO2 Thr92Ala polymorphism was linked to lower glycemic control (T2DM) (78) Dora and colleagues also found that the homozygosity of this polymorphism was associated with increased risk for T2DM in a study conducted in Brazil [(92)].

This study revealed that rs225017 SNP has no association with the glycemic profiles of the hypothyroidism patients (Table 3-14). However, Leiria and colleagues in a previous study conducted in Brazil showed that patients with T2DM with the rs225017 DIO2 polymorphism have an increased risk of developing insulin resistance [(81)].

An explanation for that the impact may differ from race to race, as previous studies conflicted about the polymorphism effect depending on the population.

4.4 The Association between DIO2 Genetic Polymorphisms and the Blood Pressure Parameters

The hypothesis was that due to D2 enzyme expression and regulatory function in vascular smooth muscle cells and because of the vasodilatory effect of T3, any genetic polymorphism in DIO2 would alter the levels of thyroid hormones parameters and may cause hypertension [63], but our results showed that the mean of T3 resides within the normal range in all the three genotype groups indicating that the polymorphism could not affect it.

As previously shown in (Table 3-15), the rs225014; T>C SNP has no effect on the blood pressure parameters, this outcome is coming along with what Maia and colleagues have found in a study conducted in Massachusetts, USA; they stated that DIO2 rs225014; T>C polymorphism was not associated with hypertensive traits [93] van der Deure and colleagues also found that in the Dutch population, there was no correlation between this polymorphism and blood pressure or the risk of hypertension [94] Canani and colleagues also found that this polymorphism was not a major determinant of blood pressure levels or hypertension in a study conducted in Brazil [95] .

However, a study by Gumieniak and colleagues conducted on patients from the USA, France, and Italy found that the C allele of this SNP increases the risk for the development of hypertension [96].

The rs225017 does not affect blood pressure parameters in the present study (Table 3-16), this again comes along with what Leiria and colleagues found in a study on the Brazilian population [81].

4.5 The Impact of the Presence of both rs225014 SNP and rs225017 SNP on the Study Parameters

To investigate the impact of the presence of both SNPs in the patients on the demographic, thyroid laboratory parameters, blood pressure and glycemic parameters the patients were divided into three groups; patients with no polymorphism, patients who have either rs225014 SNP or rs225017 SNP and patients who have both the two SNPs.

There were no significant variations in the all parameters (demographic characteristics, thyroid hormones, blood pressure and glycemic Parameters), this indicates that there is no effect for the presence of both the two SNPs on these parameters.

Conclusions

Depending on the results that obtained from this study, the followings may be concluded:

1. Two SNPs (rs225014 and rs225017) with variable allele frequencies were detected in the DIO2 gene in Iraqi hypothyroidism female patients.

2. The homozygous mutant type (CC) was the most predominant genotype regarding rs225014; 274 T>C SNP, while for rs225017; A>T, the wild type (AA) was the most frequent genotype.

3. The DIO2 gene SNPs that was detected in Iraqi hypothyroid females was noted to be non-significantly associated with most of thyroid hormones.

Recommendations and Future Work

1. Investigating other SNPs in DIO2 gene along with a larger number of the hypothyroidism patients and search their impact on the response to levothyroxine.

2. Investigating the genetic variations in thyroid hormones transporters, other types of deiodinases and TSH receptor that may contribute to variations in the therapeutic response of levothyroxine.

3. Adding a control group from the same population to certainly determine if the genetic polymorphism has a role in developing hypothyroidism.

4. Adding a male patients group from the same population.

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Appendices

Questionnaire for Hypothyroidism Patients

Demographic characterization

الاسم: رقم الهاتف:

الوزن: الطول:

Parameters	variable	Notes
Age		
Gender	Female	
Duration of treatment of levothyroxine		
Other diseases		
Other medication		
Systolic blood pressure		
Diastolic blood pressure		
Mean B.P		
BMI		
Levothyroxine dose		

Ministry of Higher Education
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University of Karbala
College of Pharmacy
Department of Postgraduate Studies

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة كربلاء
كلية الصيدلة
شعبة الدراسات العليا

العدد: د.ع/ 6 / 1396
التاريخ: 16 / 9 / 2021

Date:

امر اداري

استناداً الى الكتاب الصادر من جامعة كربلاء/امانة مجلس الجامعة ذي العدد ج/1068 في 2021/8/15 والمتضمن المصادقة على محضر الجلسة رقم (15) الثانية المفتوحة لمجلس كلية الصيدلة المنعقد بتاريخ 2021/8/3 واستنادا الى الصلاحيات المخولة لنا تقرر اقرار بحث الدراسات العليا (ماجستير) في فرع الادوية والسموم للطالبة (منتظر زيارة طعمة) والمدرجة تفاصيله في الجدول ادناه.

عنوان البحث الموسوم	الاشراف	الملاحظات
The empact of deiodinase-2 polymorphisms on the therapeutic response of levothyroxine in hypothyroidism patients of Karbala province	أ.د. بان حوشي خلف أ.م.د. سوزان جبير عباس	مشرف اول مشرف ثاني

أ.د. احمد صالح الخزعلي
عميد كلية الصيدلة
2021 / 9 / 17

نسخة منه الى:
- مكتب السيد العميد ، للتفضل بالاطلاع .
- مكتب معاون العميد للشؤون العلمية .
- شعبة الدراسات العليا للحفظ مع الاوليات .
- فرع الادوية والسموم
- فرع الكيمياء الصيدلانية.
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العراق- محافظة كربلاء- مكتب بريد كربلاء- ص ب 1125

الملخص

الخلفية: هناك ملاحظة سريرية لمرضى قصور الغدة الدرقية في السكان المحليين بأن العديد من المرضى لا يزالون يعانون من الأعراض ويشكون من المرض حتى بعد علاجهم باستخدام ليفوثيروكسين كعلاج بديل. يلعب إنزيم دي ايودنيز- النوع الثاني دورًا رئيسيًا في تحويل هرمون الغدة الدرقية الثايروكسين إلى الشكل النشط ثلاثي يودوثيرونين. تبحث هذه الدراسة في تأثير تعدد أشكال النوكليوتيدات المفردة لجين ديوديناز- النوع الثاني على الاستجابة السريرية للعلاج بالليفوثيروكسين في مرضى قصور الغدة الدرقية في محافظة كربلاء.

المنهجية: في هذه الدراسة المقطعية، تم اخذ مائة وخمسين مريضة عراقية مصابات بقصور الغدة الدرقية الأولي، كانوا المرضى بعمر 40 سنة فما فوق، وكانوا قد تم علاجهم باستخدام الليفوثيروكسين. تم تقييم هرمونات الغدة الدرقية لجميع المرضى. اما بالنسبة للتحليل الجيني فقد تم استخدام تقنية تفاعل البوليميراز المتسلسل لتضخيم البادئات الرباعية للكشف عن الطفرة rs225014. بالنسبة التغاير الجيني الاحادي rs225017 فقد تم تحليل تتابعات القطعة المضخمة باستخدام تقنية سانكر.

النتائج: توزيع الأنماط الجينية التغاير الجيني الاحادي rs225014 كان ٢٢ (١٤.٦٦٪) و ١٩ (١٢.٦٦٪) و ٧٢ (٧٢.٦٦٪) كنوع سائد TTو متغاير الزيجوت TC و متماثل الزيجوت CCعلى التوالي. كان مستوى هرمون الثيروكسين الكلي أعلى بشكل ملحوظ في مرضى متغاير الزيجوت TC منه في مرضى متماثل الزيجوت CC ، بينما لم تكن هناك فروق ذات دلالة إحصائية في مستويات هرمون تحفيز الغدة الدرقية، وهرمون ثلاثي ايودوثيرونين الكلي والحر وهرمون الثيروكسين الحر في مجموعات النوع السائد TT ومتغاير الزيجوت TC ومتماثل الزيجوت CC من المرضى. كان مستوى الأنسولين في مصل الصيام و تقييم نموذج التماثل الساكن لمقاومة الأنسولين HOMA-IR لدى مجموعة متغاير الزيجوت TC أعلى بكثير من حاملي النمط الجيني متماثل الزيجوت CC . بالنسبة لتوزيع الطرز الجينية التغاير الجيني الاحادي rs225017 كان ١٢٠ (٩٢.٣٪) و ٩ (٦.٩٪) و ١ (٠.٨٪) كنوع سائد AA و متغاير الزيجوتAT و متماثل الزيجوتTT على التوالي. كان هناك فرق معنوي في مستوى ثنائي يودوثيرونين ونسبة T3 / T4 المولية بين المجموعات الثلاث بينما لم يكن هناك ارتباط معنوي بين الأنماط الجينية المختلفة مع هرمون ثلاثي يودوثيرونين الكلي والحرو هرمون الثيروكسين الكلي والحر هرمون محفز الغدة الدرقية و هرمون ثلاثي يودوثيرونين العكسي هرمون ثنائي يودوثيرونين وجرعة الليفوثيروكسين.

الاستنتاجات: نظرًا لأن تعدد الأشكال الجينية لجين ديودينيز- النوع الثاني لا يرتبط بمعظم مستويات هرمونات الغدة الدرقية، فإنه قد لا يؤثر على الاستجابة لعلاج الليفوثيروكسين في عينتنا من مرضى قصور الغدة الدرقية ، مع ذلك فأن التغاير الجيني الاحادي rs225014 لا يمكن استبعاده من الارتباط بـمرض قصور الغدة الدرقية لأن النوع المتحول متماثل الزيجوت (CC) منه هو النمط الجيني الأكثر شيوعًا في عينة مرضى قصور الغدة الدرقية في محافظة كربلاء.

جامعة كربلاء

كلية الصيدلة

فرع الادوية والسموم

تأثير تعدد الاشكال الجينية لجين ديأيودنيز- النوع الثاني على الأستجابة العلاجية للليفوثايروكسين في مريضات قصور الغدة الدرقية في محافظة كربلاء

رسالة مقدمة الى

كلية الصيدلة في جامعة كربلاء كجزء من متطلبات درجة الماجستير في الادوية والسموم

الطالب

منتظر زيارة طعمة

(بكالوريوس صيدلة ٢٠١٦)

بإشراف

ا.د. بان حوشي خلف

ا.م. د. سوزان جبير عباس