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Role of Mitochondrial DNA in Development of Type 2 Diabetes Mellitus with/without Ischemic Heart Diseases of Iraqi Patients

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Abstract

Objectives: This study aimed to investigate the associations between various biomarkers and the specific mutation of mitochondrial DNA in type 2 diabetes mellitus with ischemic heart diseases and compared with T2DM patients without ischemic heart disease.

Methods: The study select two groups of patients admitted to Kerbala Heart Center and Al-Hassan Center for Endocrinology and Diabetes, Al-Hussein Teaching Hospital, Al-Hussein Medical City, Kerbala Health Directorates/Kerbala – Iraq between Nov., 2020 and Aug., 2021. The first group includes 50 patients of type 2 diabetes mellitus with ischemic heart disease (28 male and 22 female) with age ranged between 45–76 years, and the second group includes another 50 patients with type 2 diabetes mellitus without ischemic heart disease (24 male and 26 female) with age ranged between 49–82 years. Fasting serum glucose, insulin and insulin resistance have been determined and then correlated with nutation mitDNA investigated in sera of T2DM with/without ischemic heart diseases.

Results: The amplification of the MTLL1 gene gives one genotypes as indicated by (422 bp) bands for those with homozygous wild type (AA), homozygous mutant (GG) genotypes and two genotypes bands (422 bp) for those with heterozygous (GA). The obtained data revealed that a strong positive correlation between Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) and insulin ($r = 0.926$) with significant differences ($P < 0.05$) was obtained in the sera of type 2 diabetic patient with ischemic heart disease as compared with that obtained in type 2 diabetic patients without ischemic heart diseases.

Conclusion: The prevalence of association between HOMA-IR with MTTL1 G3243A mutation (GG allele) in type 2 diabetic patients with ischemic heart disease was only 8.0% and may be associated with maternally inherited of type 2 diabetes mellitus with ischemic heart disease as a pathogenic mutation in Iraqi population.

Keywords: Diabetes Mellitus, Type 2, Myocardial Ischemia, mitDNA, HOMA-IR, MTLL1 Gene, SA-PCR

Introduction

Diabetes mellitus, commonly known as diabetes, is a group of metabolic disorders characterized by a high blood sugar level over a prolonged period of time. One of the most important symptoms is polyuria, weight loss, constant thirst.[1] If left untreated, diabetes can cause many complications. Acute complications can include diabetic ketoacidosis, hyperosmolar hyperglycemic state, or death. Serious long-term complications include cardiovascular disease, stroke, chronic kidney disease, foot ulcers, damage to the nerves, damage to the eyes and cognitive impairment.[2]

Type 1 diabetes mellitus previously called insulin-dependent diabetes mellitus (IDDM) is caused by loss of insulin-secreting capacity due to selective autoimmune destruction of the pancreatic beta cells. Insulitis (i.e., mononuclear-cell infiltration of the pancreatic islets) is the direct result of the autoimmune process.[3]

Mitochondrial DNA comprises 0.1–2% of the total DNA in most mammalian cells. There are several unique features of the mitDNA: human mitDNA is circular, 16 kbp long, and inherited from the mother. It encodes two rRNAs, 22 tRNAs, and 13 proteins, all of which are involved in the oxidative phosphorylation process.[4] The intragenic sequence is almost absent or limited to a few bases,[5] and mitDNA does not have histones, instead it is organized in nucleoid structures. A large number of experiments showed that multiple copies of mitDNA could be found in each nucleoid, usually from two to 10 copies each, depending on the cell line studied.[6] Two different strands can be recognized in the mitDNA: the heavy strand rich in guanine bases, which also contain the majority of mitochondrial coding genes, and the light strand, encoding only for the MT-ND6 (NADH-ubiquinone oxidoreductase chain 6) protein and eight tRNAs. Both strands are transcribed at the same time, giving origin to very long transcripts, of almost mitDNA length, that are subsequently processed. Transcription seems to take place in the nucleoids due to the presence of the mitochondrial transcription machinery.

The process of mitochondrial transcription termination is still unclear. There is still a debate if MTERF1 is really needed for the termination of all the transcription processes that originate from the three different promoters of the control region. Biochemical studies have shown that MTERF1 only partially terminates H-strand transcription[7] whereas transcription in the opposite direction (L-strand transcription) is almost completely blocked.

Many different proteins are involved in the regulation of transcription, such as hormones, nuclear transcription factors, and chromatin remodeling enzymes which are also able to interact with the mitDNA, and RNA/DNA modifying enzymes. One of the first proteins investigated to be involved in the regulation of transcription is the thyroid hormone T3, which is able to promote the mitDNA transcription by directly binding the mitDNA genes.[8] Glucocorticoid hormones were also found to be in mitochondria where they modulate the transcription binding to the glucocorticoid receptor present in the mitochondrial inner membrane.[9] The estrogen receptor (ER) was found in the mitochondria of cardiac cells. It was

hypothesized that E2 (17β-estradiol) and ERβ-mediated cardioprotection was dependent on mitDNA transcription encoding for mitochondrial respiration activity. It was also demonstrated that E2 can also increase the ER β mitDNA binding activity followed by an increase in complex V encoding gene expression.[10] Melatonin was also described as a potential hormone that can control the mitDNA expression through the reduction of several mitochondrial transcription factors. It was demonstrated that melatonin was able to decrease, at both mRNA and protein levels, various proteins such as transcription factors TFB1M and TFB2M, interfering with mitDNA transcription.[10]

The mitochondrial oxidative phosphorylation system is made up of five multi-subunit enzyme complexes that are found on the inner mitochondrial membrane. The mitDNA encodes one or more of the necessary components for the NADH-ubiquinone oxidoreductase (Complex I), ubiquinone-cytochrome c oxidoreductase (Complex III), cytochrome c oxidase (Complex IV), and ATP synthase (Complex V), whereas nDNA encodes the complete succinate-ubiquinone oxidoreductase.[11,12] The mitDNA strands are known as the heavy strand (H-strand) and the light strand (L-strand), with the former being guanine rich and the latter being cytosine rich. The H-strand encodes 28 genes, whereas the L-strand encodes the remaining nine.

The A3243G mutation of the mitochondrial tRNA(Leu) gene was found to segregate with maternally inherited diabetes mellitus, sensorineural deafness, hypertrophic cardiomyopathy, or renal failure in a large pedigree of 35 affected members in four generations.[13] Presenting symptoms almost consistently involved deafness and recurrent attacks of migraine-like headaches, but the clinical course of the disease varied within and across generations. The A3243G mutation has been previously reported in association with the mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episode syndrome (MELAS) and with diabetes mellitus and deafness.[14]

The aim of the presented work is to investigate a genetic mutation of mitochondrial DNA in Iraqi type 2 diabetic patients with ischemic heart diseases of Kerbala province: Iraq, and its correlation with insulin resistance as compared with those diabetic patients without ischemic heart diseases.

Materials and Methods

The current study was cross-sectional study which includes two groups. The first group includes 50 patients of type 2 diabetes mellitus with ischemic heart disease (28 male and 22 female) with age ranged between 45–76 years., and the second group includes another 50 patients with type 2 diabetes mellitus without ischemic heart disease (24 male and 26 female) with age ranged between 49–82 years. The study was managed throughout the period between Nov., 2020–Aug. 2021. The sample collected from Kerbala Heart Center and Al-Hassan Center for Endocrinology and Diabetes, Al-Hussein Teaching Hospital, Al-Hussein Medical City, Kerbala Health Directorates/Kerbala – Iraq. The biomarker parameters investigation and molecular studies were done in the laboratories of Department of Chemistry and Biochemistry, College of Medicine, University of Kerbala and Al-Hussein Teaching Hospital laboratories'. The protocols of the study were approved by ethical committee after a verbal written informed consent for participation and for taking a blood samples for investigations from everyone enrolled in this study.

Five milliliters of blood was drawn by vein puncture from all individuals participated in this study after taking the patient's consent. The collected blood was divided into three parts, one ml of blood was used for molecular analysis, collected in EDTA containing tube and used for DNA extraction, then was analyzed directly to obtain high purity of DNA. Another one ml was placed in EDTA containing tube for analyzing HbA1c%. The remaining 3.0 ml of blood withdrawn was placed in a gel tube and left for 15 minutes at room temperature for coagulation, then centrifuged for 15 minutes at 3000 xg. Serum was collected, then frozen till analyses for determination of various biomarkers including insulin and Homeostatic Model Assessment for Insulin Resistance (HOMA-IR).

Mitochondrial DNA was extracted from whole blood that collected from patient and control groups by using DNA isolation kit "G-spinTM Total DNA Extraction Kit" (cyntol). Genotyping will be carried out by allele-specific ARMS-PCR for –3243 A/G SNP of PRKCB1 (A3243G), primers and a master mix kit (Promega) were used; PCR products were separated on a 1.0% agarose gel electrophoresis.

Two primers for allele specific-PCR designing were used for the detection of –3243 A/G polymorphism of MTLL1. The amplification-refractory mutation system (ARMS), is considered a simple, fast, and reliable technique for detecting any mutation include single base changes. ARMS is based on the use of sequence-specific PCR primers that promote amplification of test DNA only when the target allele is included within the specimen and will not amplify the non-target allele. Following an ARMS reaction the existence or absence of a PCR product is detection for the existence or absence of the target allele.

For the mutant-specific primer (M), the 3' terminal base of the ARMS primer should be complementary to the mutation sequence; for the normal-specific primer (N), the 3' terminal base should be complementary to the corresponding normal sequence.[15] The PCR reaction program procedures for SNP (–1504 C /T) in PRKCB-1 was presented in Table 1.

The 1.0% agarose solution was prepared using trisborate EDTA buffer which was diluted 1:10 with deionized water. About 4 µl of PCR product were loaded to each well with great precaution to prevent damages of the wells and cross contamination of neighboring wells. An electric field (50 V for 35 min) was established to the system causing the negatively charged nucleic acids to travel across the gel to the positive electrode (anode), and 4 µl of DNA ladder (1000 bp intron) was used as standard and band size ladder was 100–1000 bp. To visualize the DNA bands, the agarose gel was placed in the

Table 1. **The optimized PCR reaction program**

Type of cycle	Temperature ˚C	Time	No. of cycle
Intial denaturation	95	5 min	1 cycle
Denaturation	95	30 sec	
Anealing	61	30 sec	35 cycle
Extension	72	60 sec	
Final extension	72	5 min	
Total Time: 1 hour and 35 min			

UV trans illuminator device and exposed to UV light and the photos were captured by digital camera linked to PC.

Following an ARMS reaction the existence or absence of a PCR product is detection for the existence or absence of the target allele. For the mutant-specific primer (M), the 3' terminal base of the ARMS primer should be complementary to the mutation sequence; for the normal-specific primer (N), the 3' terminal base should be complementary to the corresponding normal sequence.[16]

Total 422 nucleotides containing DNA was amplified by polymerase chain reaction (PCR), The forward primer was taken from nucleotide sequence 3035 to 3054 as 5'- GCA AGA GAT ACA GTG TTG CTC CA -3' and the reverse primer was taken from nucleotide sequence 3437 to 3456 as 5'- CGT TCT CTA TGT CAC AAC GAG GT-3' After electrophoresis, absence of the mutation generates a single band (422 bp).

The amplification product of MTTL1 gene polymorphism (SNP of A3243G) detected by allele specific PCR reaction, have a size of 422 bp. The PCR product was electrophoresed on 1.0% agarose and directly was visualized with ethidium bromide under UV light. The optimization of PCR assay constituents used was indicated in Table 2. Green master mix is a (ready-to-use solution) encompass bacterially derived *Taq* DNA polymerase, dNTPs, $MgCl_2$ and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR.

Fifty ml of 1% agarose solution was prepared in 10X TBE buffer and three µl of ethidium bromide was added to the solution. The gel solution was poured into the chamber and permitted to be harden for approximately 30 minutes at room temperature. Then combs were removed, and then samples and DNA ladder were loaded (4 µl of 1000 bp, intron was used as standard and band size ladder was 100–1000 bp) on each well with extreme cautions to avoid damages of the wells and cross contamination of neighboring wells and then placed in a horizontal electrophoresis system and covered with the same TBE buffer that used to prepare the gel. The cathode (black) was connected to the wells side of the unit and the anode (red) to the other side. Electrophoresis is attach to direct current power source until dye markers migrated to the suitable distance, according to the size of DNA fragment that recognized. About 4 µl of PCR product were loaded to each well with great precaution to prevent damages of the wells and cross contamination of neighboring wells. An electric field (50 V for 35 min) was established to the system causing the negatively charged nucleic acids to travel across the gel to the positive electrode (anode). To visualize the DNA bands, the agarose gel was placed in the UV trans illuminator device and exposed to UV light and the photos were captured by digital camera linked to PC.

Table 2. **Optimization constituents of PCR components used**

Reagent	Volume
Forward primer	2 µl
Reverse primer	2 µl
Master mix	10 µl
Nuclease free water	5 µl
DNA template	5 µl
Total volume	25 µl

Results and Discussion

During the current cross-sectional study, 50 of cases include T2DM patients with ischemic heart disease and another 50 diabetic patients without ischemic heart disease groups was enrolled. The ANOVA test found that there was a non-significant difference in age between diabetic patient have IHD and without IHD groups. This age matching helps to eliminate differences in parameters, Figure 1.

All patient group study comprised of 46 females and 54 males, this result was different with Maas and Appelman et al.[17] who was found that cardiovascular disease effects women 7 to 10 years later than it does males, yet it remains the leading cause of mortality in women, due to the misunderstanding that women are "protected" from cardiovascular disease, the risk of heart disease in women is frequently overestimated. Because of the under-recognition of cardiac disease and the variations in clinical presentation between men and women, less aggressive treatment options are used and women are neglected in clinical trials. Furthermore, women's self-awareness and identification of their cardiovascular risk factors require more attention, which should lead to better cardiovascular event prevention, Table 3.[18]

The mean ± SD of BMI in type 2 diabetic patients without IHD was (31.6818 ± 4.42 kg/m^2) which was non-significantly higher than that found in type 2 diabetic patients with IHD (30.0903 ± 4.99 kg/m^2) and the ($P > 0.05$). Some study demonstrated that obesity is a complex metabolic condition reported that affects 35% of the adult population in the United States, according to the National Institutes of Health. As a significant risk factor for ischemic heart disease (IHD) and its metabolic consequences, it has elevated to become one of the most serious health problems in many regions of the world.[19]

It was not noticed that a significant differences in body BMI between type 2 diabetic patients with/without heart disease and this results indicates the size of the sample used and the time obtained for blood samples. Although there are most studies that indicate a significant clinical relationship between body weight and heart disease.[20]

The mean ± SD of HbA1c% in type 2 diabetic patients with ischemic heart disease was (9.674 ± 1.72%) which was slightly non-significantly higher than that found in patients without ischemic heart disease (9.64 ± 2.087%) ($P = 0.921$), this data was disagreement with other study which found that HbA1c was associated with cardiovascular disease (CVD), such as carotid and coronary artery atherosclerosis, ischemic heart disease, ischemic stroke, and hypertension, among other

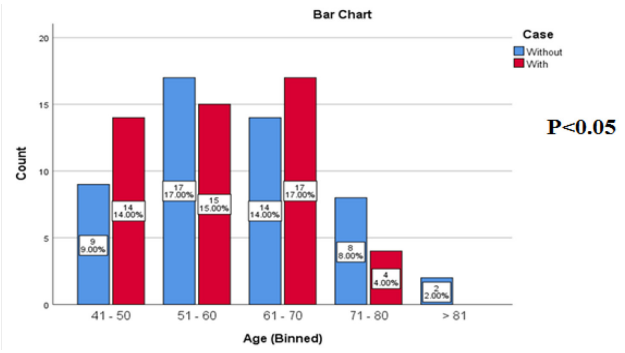

Fig. 1 **Number and percentage of age in T2DM with and without IHD groups.**

Table 3. **Number and percentage of diabetic patient with IHD and without non-IHD according to their gender status**

Gender	T2DM cases		Total	*P*-value
	Without IHD	With IHD		
Female	22	24	46	
	44.0%	48.0%	46.0%	
Male	28	26	54	0.421
	56.0%	52.0%	54.0%	
Total	50	50	100	

Table 4. **Show the level of HbA1c%, insulin, HOMA-IR and blood glucose concentration in T2DM with/without ischemic heart disease**

		N	Mean ± SD	*P*-value
HbA1c%	Without	50	9.64 ± 2.087	0.921
	With	50	9.674 ± 1.72	
Insulin, µU/mL	Without	50	6.03 ± 5.234	0.392
	With	50	6.86 ± 4.31	
HOMA-IR	Without	50	2.65 ± 2.41	0.145
	With	50	3.351 ± 2.38	
FBG, mg/dL	Without	50	185.5 ± 56.77	0.184
	With	50	198.9 ± 42.283	

things. HbA1c causes dyslipidemia, hyperhomo-cysteinemia, and hypertension, as well as an increase in C-reactive protein level, oxidative stress, and blood viscosity, all of which are associated with the development of cardiovascular illnesses.[21]

The mean ± SD of insulin level determined in both group of diabetic patients studied indicated that its level in type 2 diabetic patients with ischemic heart disease was (6.86 ± 4.31 µU/mL) which non-significantly higher than that found in type 2 diabetic patients without ischemic heart disease is (6.03 ± 5.234 µU/mL) with $P > 0.05$, Table 4. Cardiovascular illnesses are the leading cause of death worldwide,[22] and type 2 diabetes is one of them because it is so common and doubles the risk of heart disease. Increased glucose and insulin concentrations, as a result, have been proven to be pro-atherogenic causes,[23] whereas other study showed that cardiovascular diseases may be a consequence of insulin resistance rather than being caused by toxic effects of high insulin or glucose concentrations, Table 4.[24]

The level of HOMA-IR found in type 2 diabetic patients with ischemic heart disease was (3.351 ± 2.38) but in diabetic patients without ischemic heart disease were (2.65 ± 2.41) and the results is a non-significant (*P*-value > 0.05), Table 4. Assessment of the homeostasis model insulin resistance (HOMA-IR) is a widely used and validated diagnostic of insulin resistance that includes both glucose and insulin concentrations. Insulin resistance, increase the risk of atherosclerosis through a variety of pathways[25] and it has been linked to coronary artery disease.

Table 4 also shows the results concerning the fasting blood glucose level in sera of type 2 diabetic patients with/without ischemic heart diseases. The mean ± SD of FBG level determined in both group of patients studied indicated that its level in type 2 diabetic patients with ischemic heart disease was (198.9 ± 42.283 mg/dL) which is non-significantly higher than that observed in T2DM without IHD (185.5 ± 56.77 mg/dL), ($P > 0.05$). The impact of hyperglycemia on coronary heart disease (CHD),[26] stroke,[27] and other cardiovascular diseases (CVDs)[28] has been widely studied. In people with hyperglycemia, two-hour plasma glucose (2hPG) is a better predictor of coronary heart disease (CHD) and ischemic stroke (IS) than fasting plasma glucose (FPG), but nothing is known regarding their impact in the normoglycemic range. Insulin resistance and beta cell dysfunction are already evident in people with increased normal FPG, Table 4.[29]

The molecular investigations concerning mitochondrial DNA found just four cases has G allele in patient have Ischemic heart disease, and one case without Ischemic heart disease as in the Figure 2. The study showed that the G allele is responsible for heart disease, an individual's carrying the MTTL1 A3243G mutation have been diagnosed with ischemic cardiac disease.[30] The mutation affects mitochondrial DNA structure, stability, methylation, amino-acylation, and codon recognition capabilities, making it difficult to couple the mRNA codon with the mutant tRNA anticodon.[31] This condition is most commonly linked to an A to G transition in the mitochondrial DNA at location 3243. Incorrect RNA processing results in reduced translation as well as decreased rates of protein synthesis and enzyme activity. A statistically significant negative relation was observed between the frequency of MTTL1 A3243G mutations and the particular activity of the mitochondrial respiratory chain complex (Figure 2).

The observed results found that the mean ± SD of HOMA-IR in (38/50) of type 2 diabetic patient without ischemic heart disease have predominantly AA allele of MTTL1 A3243G mutation (38/50) in their blood (2.881 ± 1.64), and (16/50) of type 2 diabetic patient with ischemic heart disease was (3.344 ± 1.951) whereas, only one type 2 diabetic patient without IHD indicate HOMA-IR have GG allele of MTTL1 A3243G mutation in his blood (1.114 ± 0.031). On the other hand, only (4/50, 8%) of type 2 diabetic patients with IHD have GG allele of MTTL1 A3243G mutation and the HOMA-IR observed was (4.24 ± 1.76) Table 5.

The genotype of GG allele in multigenerational impact of the MTTL1 A3243G increases the risk of HOMA-IR in mitochondria and their genome are found in the cytoplasm of cells, with thousands of copies of the mitochondrial genome in most cell types. Heteroplasmy occurs when not all copies of the mitochondrial genome have the same sequence at the tissue or even cellular level with resulting in different proportions of mutant and wild type mitochondria.[32]

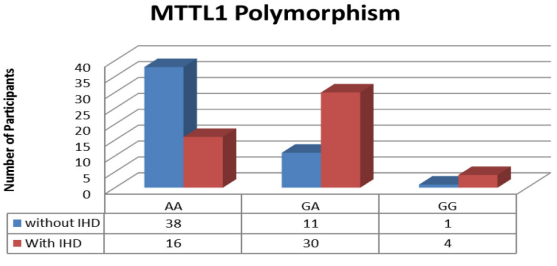

Fig. 2 **The relationship between multigenerational impact of the MTTL1 A3243G with homeostatic model assessment for insulin resistance.**

Table 5. **The relationship between multigenerational impacts of the MTTL1 A3243G with HOMA-IR**

T2DM	Number of patients and percentage	MTTl1 Gene	HOMA-IR Mean ± SD	*P*-value
Without IHD, *N* = 50	38/50 (76%)	AA	2.881 ± 1.64	0.013
	11/50 (22%)	GA	1.974 ± 1.33	
	1/50 (2%)	GG	1.114 ± 0.031	
With IHD, *N* = 50	16/50 (32%)	AA	3.344 ± 1.951	
	30/50 (60%)	GA	3.236 ± 2.68	
	4/50 (8%)	GG	4.24 ± 1.76	

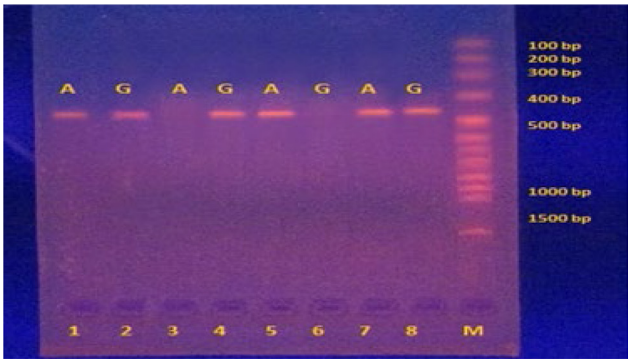

Fig. 3 **The electrophoresis profiles for some of the successful amplifications. Multigenerational Impact of the MTTL1 A3243G. M = Lane for DNA ladder marker; 1,2 = Lane for hetrozygote patient; 3,4 = Lane for G allele patient; 5,6 = Lane for A allele patient 7,8 = Lane for hetrozygote patient.**

When mitochondria are randomly segregated to each new cell during cell division, the fraction of mitochondrial DNA containing a mutation may vary between daughter cells as a result of heteroplasmy. When the load or proportion of mitochondrial DNA with a harmful mutation exceeds a certain threshold level, tissues show pathogenic effects of mutation.[33]

As illustrated in Figure 3, the successful amplification and analysis of the SNP of Multigenerational Impact of the MTTL1 A3243G was achieved using the A3243G. Detection of PCR bands of suitable size in the 1% agarose gel indicated that the samples were of the appropriate genotype. The amplification product of MTTL1 gene polymorphism (SNP of A3243G) detected by allele specific PCR reaction, have a size of 422 bp.

The observed data concerning mitDNA was disagreed with other study which screened 142 patients of type 2 diabetes mellitus and 142 healthy control individuals to detect 3243 A/G mutation, their ages of the onset for type 2 diabetes mellitus varied from 34 to 52 years. They found a disappearance of 3243 A/G mutation in type 2 diabetes patients and healthy control individuals. The levels of fasting and postprandial glucose indicate severe hyperglycemia, resulted in high glycosylation of hemoglobin (HbA1c),[34] whereas, in this study the 3243 A/G mutation it is appeared in type 2 diabetes mellitus with ischemic heart disease.

Conclusion

According the observed results we can conclude that the prevalence of association between HOMA-IR with MTTL1 G3243A mutation (GG allele) in type 2 diabetic patients with ischemic heart disease was only 8.0% and may be associated with maternally inherited of type 2 diabetes mellitus with ischemic heart disease as a pathogenic mutation in Iraqi population.

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