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Assessment of *HNF4A* Gene Expression as a Potential Biomarker for T2DM Predicting and its Correlation with Biochemical Parameters in Iraqi Population

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Abstract: Type 2 diabetes mellitus (T2DM) is a complicated disease that is caused by a combination of genetics and environment. The hepatocyte nuclear factor (*HNF4A*) is responsible for regulating hepatic gluconeogenesis and insulin production. Several studies have connected the corresponding gene to type 2 diabetes. This study involved the collection of 150 blood samples, which were divided into three groups: fifty youthful patients (10-35 years) and fifty elderly patients (40-85 years) with a previous diagnosis of T2DM. Additionally, fifty blood samples from healthy, typical individuals have been collected. All of the participants had their complete medical histories taken, received a comprehensive clinical examination, and had standard laboratory tests performed, such as blood sugar levels while fasting, hemoglobin A1c levels, and total cholesterol. Also, the gene expression of *HNF4A* in comparison with housekeeping gene *GAPDH* has been done. The results showed that levels of fasting serum glucose, hemoglobin A1c and total cholesterol had a significant increase in T2DM patients as compared to control group. The analysis of *HNF4A* gene expression revealed that there was no statistically significant difference for gene expression of the *HNF4A* in compression with control. The study conclude that a significant correlation existed between *HNF4A* gene expression with the biochemical tests.

Key words: Type 2 diabetes mellitus, T2DM, hepatocyte nuclear factor, *HNF4A*, biochemical tests.

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Introduction

Diabetes mellitus (DM) is a syndrome with multiple etiologies that is primarily characterized by chronic hyperglycemia and metabolic dysfunctions involving proteins and lipids. Hyperglycemia may be associated with the inability to produce, secrete, or assimilate insulin, or with a combination of all of these abnormalities (1). According to the International Diabetes Federation. Type 2 diabetes is the most prevalent kind of the disease, accounting for 85 to 95% of cases in developed nations and an even larger number in underdeveloped countries. T2DM is becoming more common globally, particularly in Iraq, where it has become a significant health issue (2). Research in recent years has concentrated on finding possible biomarkers for the diagnosis and treatment of T2DM (3). Type 2 diabetes mellitus which disrupts glucose homeostasis, is a global health issue (4). Over the past two decades, the T2DM incidence has increased dramatically, causing critical societal and economic costs. About 537 million 20–79-year-olds have diabetes, according to the International Diabetes Federation, this number will increase to 643 million by

2030 and 783 million by 2045. Type 2 diabetes occurs when peripheral tissues become insulin resistant (5). Insulin deficit occurs when the pancreas cannot generate enough insulin to overcome this resistance (6). At least two pathological mechanisms are involved: (a) a progressive decline in pancreatic islet cell activity resulting in reduced insulin output and inadequate glucagon suppression. (b) peripheral insulin resistance, which reduces insulin metabolism. Non-insulin dependent diabetes mellitus (NIDDM) and adult-onset diabetes are other names for type 2 diabetes. Children are not excluded from developing this condition, and some patients need insulin medication, therefore these labels are misleading. Type 2 diabetes is multifactorial disease (7). Anxiety, stress, advanced age, obesity, sedentary lifestyle, irregular food and many other factors could cause it. Obesity causes 55% of type II diabetes and lowers saturated fat intake. It is a genetic-environmental illness (8). Most genetic variables underlying the illness are unknown. Monogenic maturity-onset diabetes of the young (MODY) accounts for 1–2% of diabetes cases. It is characterized by early onset (typically adolescent or infancy), dominant inheritance, and β-cell dysfunction. MODY1 is the form of MODY caused by mutations in the hepatocyte nuclear factor-4-alpha (*HNF4A)* transcription factor. Genetic linkage studies have shown that MODY1 is closely associated with loci near *HNF4A* on chromosome 20 (9). Previous study mentioned that *HNF4A* is the most abundant DNA-binding protein in the liver and controls genes predominantly engaged in the hepatic gluconeogenic pathway and lipid metabolism. The gene *HNF4A* is a member of the nuclear receptor family of transcription factors. It is needed for glucose metabolism and proper insulin gene expression and release in pancreatic β-cells and hepatic gluconeogenesis. Pancreatic β-cell insulin secretion impairment may induce MODY1. The deficiency in *HNF4A* may cause β-cell dysfunction (10). The *HNF4A* gene may contribute to insulin secretion problems in T2DM and MODY1 patients (11). According previous studies, it is unknown how common variations of *HNF4A* affect the chance of developing T2DM (11, 12). Rare variations within *HNF4A*, however, it have been shown to have a role in the development of T2DM (14).Therefore, the aim of the present study is to evaluate the association between *HNF4A* gene expression levels in T2DM patients in a comparison with healthy control group of a sample of Iraqi population. And to evaluate the correlation of *HNF4A* gene expression with some biochemical tests (HbA1c, fasting blood sugar and cholesterol).

Materials and methods

Study participants and design

This study included collection 150 peripheral blood samples from patients of various ages, as well as healthy control participants. Patient groups were chosen from Baghdad's private clinics. All individuals who volunteered to take part in the research provided their informed consent. All participants were adequately informed of the study's aim. The first group was an old patients' group**;** fifty blood samples were obtained from elderly Iraqi patients who had T2DM in the past. Their ages ranged from 40 to 85 years old. The second group was the young patients' group; fifty blood samples were obtained from young Iraqi patients who had previously diagnosed with T2DM. Their ages ranged from 10 to 35 years old. The third group was the healthy control group which included fifty

blood samples obtained from participants who seem to be in good health. They were chosen randomly from those who regularly visit the clinics for checks as well as from among friends and coworkers. All respondents completed a questionnaire that collected data on their age, gender, BMI, and family medical history. The three groups were classified according to family history, age, BMI and gender. The T2DM Inclusion criteria used to identify diabetes in those people who had been classified by physicians as having T2DM was based on the criteria of the American Diabetes Association. In addition to having diabetic symptoms, an individual was considered to have diabetes if his or her fasting glucose level was >126 mg/dl (8.0 mmol/l). The T2DM Exclusion criteria was any T2DM patient who had chronic kidney, liver, thyroid, or other endocrine illnesses, alcoholics and pregnant women. All these conditions were excluded from this research.

Sample collection and biochemical tests

Venipuncture was used to draw 5 ml of blood samples while maintaining aseptic conditions. Each sample was divided into three sections: first EDTA tube (2 ml); Two ml of blood was placed in an EDTA tube and kept at -20 Cº to be used in the molecular genetic study and measure HbA1C. The second tube was an Eppendorf tube, which was prepared by adding 250 µl of blood from the first EDTA tube to 750 µl of TRIzol LS reagent, then gently mixed and kept at -20 Cº to be used for gene expression tests. The third tube was prepared by adding the remaining three milliliters of venous blood a clot activator and gel serum separation tubes. serum was obtained by placing blood samples in a gel tube and allowing them to stand at room temperature (20 to 25 ℃). The serum was then separated by centrifugation at 3000 rpm per minute for 15 minutes. Serum was utilized in biochemical testing.

The biochemical tests were conducted by using routine clinical assays and a colorimetric method, following the instructions of manufacturing kits companies; Glucose kit (Linear Chemicals, Spain), Ichroma™ HbA1c Glycosylated hemoglobin kit (Biotek med,Spain) and Cholesterol MR kit (Linear Chemicals, Spain).

RNA extraction and cDNA Synthesis

Total RNA was extracted using the TransZol Up Plus RNA Kit (TransGen, biotech- China) as specified by the manufacturer. Using a NanoDrop One C spectrophotometer, the quantity and quality of the isolated RNA were determined. For RT-PCR, total RNA extracts with an OD260/OD280 ratio of were processed. The cDNA was prepared according to the manufacturer's instructions using the EasyScript®One-Step gDNA Removal and cDNA Synthesis SuperMix reagent (TransGen biotech-China). cDNA was kept until it was used as a template for RT-PCR.

Assay for Real-Time RT-PCR

To measure the threshold cycle (Ct), RT-PCR reaction mixtures were prepared with (2xTransStart® Top Green qPCR Super Mix) and performed on the Qiagen Rotor gene Real Time PCR System. Each reaction has been performed individually. Every reaction was carried out twice. The housekeeping gene *GAPDH* was used as a reference gene. The gene expression reaction for each of *GAPDH* and *HNF4A* was performed separately under the following conditions: Enzyme activation at 94 ºC for 30 sec, followed by 35 cycles of 95 ºC for 5 sec, annealing temperature 58 ºC for 15 sec,

72 ºC for 20 sec and then fluorescence was measured. The primers were provided by Alpha DNA company-Canada. The sequences of the primers are listed in (table 1). Quantification of expressed gene as relative mRNA level compared with healthy control level, was calculated after normalization to *GAPDH* according to Livak method.

Table-1: Primers used in gene expression.

gene	primer	Primer sequence (5′→3′)	length	Product size bp	reference
GAPDH	Forward	GAAATCCCATCACCATCTTCCAGG	24	160	(15)
	Reverse	GAGCCCCAGCCTTCTCCATG	20		
HNF4A	Forward	CTGAGCGATCCAGGGAAGAT	20	161	This study
	Reverse	ATCTGCTCGATCATCTGCCA	20		

Statistical analysis

The data was presented as means ± SEM. The program that has been used for the statistical analysis was SPSS 18.0 (SPSS Inc., Chicago, USA). Statistics were judged significant at p-value < 0.05. One-way ANOVA was used to statistically analyze the differences between the mean values of the control participants and T2DM patients. Correlation analysis was conducted using the Pearson and Spearman correlation tests. According to Duncan's multiple range comparisons (DMRTs), means followed different letters are significantly different, while those that are followed by the same letter are not significantly different.

Results and discussion

The current study included measuring some biochemical tests of T2DM patients' groups and healthy control group. The results of fasting blood glucose showed that there was a high significant difference between these three groups with p-value (0.001), and the old patient's group had highest difference from control in comparison with young patients (Table 2). The means of fasting blood glucose levels for the old patients, young patients and healthy control were (310.7600 ± 110.42988), (217.3600 ± 134.53787) and (82.7400 ± 5.01756) mg/dl respectively. The results of HbA1c showed that there was high significant difference between the three groups with p-value (0.0001) (Table 3). The old and young patient's group had similar means of HbA1c levels in their blood about (9.9786 ±1.67732) and (9.7976 ±1.50369) mg/dl respectively, in comparison with control group (5.0856 ± 0.34445) mg/dl.

The results of cholesterol test showed that there was a high significant difference between the three groups with p-value (0.0001) (Table 4). The old and young patient's group had similar means of HbA1c levels in their blood about (203.9600 ± 66.66057) and (188.5200 ± 59.12361) mg/dl respectively, in comparison with control group (133.3000 ±9.69378) mg/dl.

Table-2: Statistical analysis of fasting blood sugar test between T2DM patients' groups and healthy control group.

Groups	Mean	Std. Deviation	Std. Error of Mean	p-value
Young Patients	217.3600 b	134.53787	19.02653	0.001**
Old Patients	310.7600 a	110.42988	15.61714	
Control	82.7400 c	5.01756	0.70959	

Table-3: Statistical analysis of HbA1c test between T2DM patients' groups and healthy control group.

Groups	Mean	Std. Deviation	Std. Error of Mean	p-value
Young Patients	9.7976 a	1.50369	.21265	0.0001**
Old Patients	9.9786 a	1.67732	20.58196	
Control	5.0856 b	0.34445	.04871	

Table-4: Statistical analysis of Cholesterol test between T2DM patients' groups and healthy control group.

Groups	Mean	Std. Deviation	Std. Error of Mean	p-value
Young Patients	188.5200 a	59.12361	8.36134	0.0001**
Old Patients	203.9600 a	66.66057	9.42723	
Control	133.3000 b	9.69378	1.37091	

The gene expression test has been done to measure the gene expression of *HNF4A* in comparison with housekeeping gene *GAPDH.* The results have been statistically analysis and the fold of *HNF4A* gene expression was illustrated in (Table 5).

Table-5: Gene expression of *HNF4A* and *GAPDH* in the T2DM patients' groups and healthy control group

Groups	Means Ct of *HNF4A*	Means Ct of *GAPDH*	ΔCt (Means Ct of *HNF4A)*	ΔΔCt	Fold of *HNF4A* gene expression $2^{-\Delta\Delta Ct}$	*p-value*
Young Patients	27.4138	16.5794	10.8344	2.89	0.19 c	0.0001**
Old Patients	25.4636	16.6292	8.8344	0.89	0.53 b	
Control	24.8028	16.8614	7.9414	0	1.00 a	

The results showed that there was significant difference in *HNF4A* gene expression between control and patients' groups (p-value = 0.0001). The *HNF4A* gene expression was higher in the healthy control in comparison to patients' groups with fold (1.00), and there was decrease in it in the diabetes patients' groups. The young patients had the less gene expression between the three groups with fold (0.19). while the fold of the old patients was 0.53. The correlation analysis for our results has been done to find the relationship between the gene expression of *HNF4A* and the biochemical tests (fasting blood sugar, HbA1c and cholesterol), the results is illustrated in (Table 6).

Table-6: Correlation of *HNF4A* with biochemical tests.

		Age	Fold of *HNF4A*	Fasting blood sugar	HbA1c1	Cholesterol
Age	***R***	1	.143	.257**	.054	.121
	P		.081	.001	.514	.141
Fold of *HNF4A*	***R***	.143	1	-.198*	-.375**	-.204*
	P	.081		.015	.000	.012
Fasting blood sugar	***R***	.257**	-.198*	1	.721**	.446**
	P	.001	.015		.000	.000
HbA1c1	***R***	.054	-.375**	.721**	1	.496**
	P	.514	.000	.000		.000
Cholesterol	***R***	.121	-.204*	.446**	.496**	1
	P	.141	.012	.000	.000	

** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

The results of (Table 6) showed that there was a highly significant negative correlation between the fold of *HNF4A* and the HbA1c1 (R= -0.375**). Also, there was a significant negative correlation between the fold of *HNF4A* and both of blood sugar and cholesterol, with R (-0.198*) and (-0.204*) respectively. The results also revealed that there was a significant positive correlation between the biochemical tests. The age had correlation with blood sugar (R= 0.257**). Glucose is the main resource that cells need for their metabolism. However, glucose cannot enter the cell without insulin (16). The pancreas, when functioning properly, produces enough insulin to get glucose into cells. Pancreatic dysfunction results in either inadequate insulin production or cellular resistance to insulin. This leads to an increase in blood glucose levels and the development of diabetes mellitus (17). According to the findings of our research, individuals with type 2 diabetes had much higher levels of glucose than the controls. This disease could be triggered by weak cells, a lack of insulin production and/or function, and increasing insulin resistance. These findings are consistent with previous study who mentioned that the blood glucose concentration of T2DM patients was noticeably greater than that of normal healthy control. Over the course of the RBC's lifespan, which is around 120 days, hemoglobin A1C has been shown to be correlated with blood glucose levels (18). Glycated hemoglobin (HbA1c) was formerly thought to be the most reliable indicator for diagnosing and managing diabetes, particularly type 2 diabetes. Normal non-diabetic HbA1c readings range from 4.0 to 5.6 percent. People with HbA1c values of 5.7 percent to 6.4 percent have prediabetes, whereas those with levels of 6.4 percent or above have diabetes (19). This study revealed a considerable increase in total HbA1C

levels between diabetic patients and healthy controls. These findings concur with those of (20) who stated that HbA1c is elevated in diabetics. Our results indicated that diabetes patients' cholesterol levels were substantially greater than those of the control group. This rise might be caused by an increase in plasma concentrations of VLDL and LDL, which could be due to increase in hepatic synthesis of VLDL or a reduction in the removal of VLDL and LDL from circulation. Blood cholesterol levels depend on a number of risk factors, including age, gender, family history, smoking, high blood pressure, physical inactivity, obesity, and diabetes. The normal range for total cholesterol is less than 200 mg/dl (21).The results of *HNF4A* gene expression revealed that there was significant difference between control and patients' groups (p-value = 0.0001). The *HNF4A* gene expression was higher in the healthy control group in comparison to T2DM patients' groups. There was significant reduction of *HNF4A* gene expression in the T2DM patients' groups, the young patients had the less gene expression among the three groups. This might be explained by the fact that any malfunction in the gene *HNF4A*, such as the presence of SNPs, is associated with a higher risk of developing type 2 diabetes. This gene is crucial for maintaining glucose homeostasis and directly activates the development of the gene insulin. *HNF4A* encodes a transcription factor that controls the expression of genes implicated in insulin secretion and glucose regulation. Many previous studies have linked it to T2DM in Caucasian and Asian populations (22). Additionally, *HNF4A* has been linked to the regulation of glucose transport and metabolism. This gene can be disrupted to cause maturity-onset diabetes of the young (MODY), a rare, autosomal dominant, noninsulin-dependent form of diabetes (23). Previous study mentioned that *HNF4A* variants are associated with MODY and/or type 2 diabetes (T2DM) (24). Mutations in the P2 promoter region have been identified as a candidate gene for type2 diabetes (22). In addition, a common polymorphism in the P2 promoter and the T130I mutation are associated with late-onset type 2 diabetes in Japanese (25). Many studies have found a correlation between *HNF4A* and T2DM in multiple ancestry populations. However, evidence suggests that disruption of this factor's function not only compromises pancreatic islet development and function (in the case of MODY), but also increases susceptibility to T2DM (26). Previous research has shown that heterozygous mutations in the *HNF1A* and *HNF4A* genes result in beta-cell dysfunction and a progressive form of hyperglycemia with diabetes that is linked to late diabetic complications (27). Our results revealed that there was significant negative correlation with *HNF4A* gene expression and the biochemical tests (Fasting blood sugar, HbA1c1and Cholesterol). Due to the role of gene *HNF4A* as a significant regulatory factor in the liver's tissues, mutations in this gene may result in a pleiotropic phenotype, such as an impairment in the liver's cholesterol and lipoprotein metabolism. Recent research indicates that a mutation in the *HNF4A* gene affects triglyceride metabolism (21). In addition, clinical investigations have demonstrated that *HNF4A* mutations are associated with impaired pancreatic and beta cell function, which is characterized by aberrant insulin and glucagon secretion (28). According to another study, there is scant evidence that *HNF4A* variants contribute

significantly to the risk of T2DM in the general population, but a modest contribution cannot be ruled out. In addition, the observation of certain mutations in control suggests that they are not highly penetrant variants that cause MODY (24). A low protein diet is associated with impaired fetal growth, adiposity, insulin resistance, and diabetes in progeny (29). During pregnancy and lactation, maternal protein malnutrition could results in progressive epigenetic silencing at the enhancer region of *HNF4A*, which weakens the promoter-enhancer interaction and causes a persistent decrease in *HNF4A* expression (30). Many of the rare mutations in *HNF4A* have only been identified in a handful of individuals, and they only rarely segregate completely with MODY. Due to the similarity of phenotypes, the proportion of T2DM in the general population attributable to MODY remains unknown. Several years after the identification of *HNF4A* as the gene responsible for MODY, evidence is accumulating that *HNF4A* may also play a role in cases of T2DM (24). These findings suggest that *HNF4A* plays a crucial role in the pathogenesis of T2DM and may serve as a biomarker for the prediction and management of T2DM.

Conclusion

The study concluded that there was a significant correlation between *HNF4A* gene expression and the biochemical tests that had been studied, and the gene expression of the *HNF4A* may be a prospective biomarker for predicting the development of type 2 diabetes mellitus. This conclusion was reached as a result of the research that was conducted.

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