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Original Article

Long non-coding *RNA-H19* and *miRNA-29a* expression in type 2 diabetes mellitus patients

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Abstract

Diabetes mellitus type 2 is one of the most prevalent illnesses. This study aims to shed light on the expression levels of the long non-coding RNA *H19* and *miRNA-29a*, which are important in the diagnosis and pathogenesis of T2DM. The study included the collection of samples from 100 patients with type 2 diabetes attending AL-Furat General Hospital in Baghdad, following medical investigation, and 100 apparently healthy individuals as a control group, from July 2023 to November 2023. The Relative *H19* expression levels were increased in the patient's group (fold 1.38) *vs.* controls (fold 1.00) with a highly significant difference ($p \leq 0.01$), similar to *miRNA29-a* levels, which were also increased in the patient's group (fold 2.17) with a highly significant difference ($p < 0.01$). The analysis of *H19* revealed an area under the curve of 0.86 ($p < 0.001$), with 68% sensitivity and 96% specificity. The positive predictive value was 84.6%. The results of the analysis of *miRNA-29a* showed that the area under the curve was 0.91 and $p < 0.001$, with 86% sensitivity and 92% specificity. The positive predictive value was 98%. The severity of diabetes 2 patients is linked to high *lncRNA H19* expression and increased *miRNA-29a* expression, suggesting a potential role for *miRNA-29a* in the disease's etiology.

Keywords: *lncRNA H19*, *miRNA29-a*, q PCR, T2DM

Introduction

Type-2 diabetes mellitus (T2DM) is a multifaceted condition characterized by diminished β-cell mass in pancreatic islets and compromised insulin release (Luo *et al.*, 2020) [1]. Type 2 diabetes mellitus (T2DM) is also identified as a metabolic disorder characterized by low-grade inflammation [2]. It is a progressive ailment marked by elevated blood sugar levels (hyperglycemia), where the body either resists insulin's effects or produces insufficient insulin [3]. Type 2 diabetes (T2DM) is a metabolic disorder usually in adults, resulting from insulin insufficiency or dysfunction [4]. Research suggests a positive association between NCSA, high BMI values, and type 2 diabetes mellitus (T2DM) in both genders [5]. T2DM induces heightened lipid peroxidation, leading to the onset of chronic complications due to oxidative stress [6]. The cell-free non-coding RNAs and microRNAs (miRNAs) have been demonstrated to serve as important diagnostic/prognostic biomarkers in diabetes [7]. The *micro*RNA-29 family members *miR-29a*-3p, *miR-29b*-3p, and *miR-29c*-3p are ubiquitously expressed and consistently increased in various tissues and cell types in conditions of metabolic disease, obesity, insulin resistance, and type 2 diabetes. In pancreatic β cells, *miR-29a* is required for normal exocytosis, but increased levels are associated with impaired β-cell function, and overexpression of *miR-29a* leads to insulin resistance [8]. Long non-coding RNAs (lncRNAs) represent a relatively understudied class of transcripts that growing evidence implicates in the pathogenesis of diabetes. ncRNAs regulate the expression of

Table 1: Primers used in gene expression.

Description	Sequence (5′→3′ direction)	Company and country
	lncRNA H19 gene	
Forward	5'ATCGGTGCCTCAGCGTTCGG3'	
Reverse	5'-CTGTCCTCGCCGTCACACC-3'	Alpha DNA company Canada
	***GAPDH* (housekeeping gene)**	
Forward	'5TGAGAAGTATGACAACAGCC'3	
Reverse	'5-TCCTTCCACGATACCAAAG-'3	

neighboring β-cell-specific transcription factors, with the knockdown or overexpression of lncRNAs impacting a network of other key genes and pathways [9]. lncRNAs may play a role in either suppressing or exacerbating diabetes-associated vascular [10].

Material and methods

Participants

This case-control study enrolled patients with type 2 diabetes mellitus (T2DM) attending AL-Furat General Hospital in Baghdad after a medical investigation between July 2023 and November 2023. One hundred samples were randomly assigned to T2DM adult patients with a confirmed diagnosis (n=50) and 50 healthy controls. The patients' information consists of age, gender, name, duration, insulin use, hypertension, hyperlipidemia, genetic factors, and type of drugs.

Sample collection and biochemical tests

Venous blood (5 ml) was collected from each subject. Each sample was divided into three parts. The 1st part (2 ml) of whole blood was transferred into an EDTA tube for HbA1c and c-peptide, the 2nd part (250 µl) was transferred into a 1.5 ml Eppendorf tube containing 750 µl of triazole reagent, which was stored in a deep freeze until the next day for the extraction of total RNA. The third remaining whole blood was placed in a Gel tube, then centrifuged for 10 minutes at 3000 rpm to separate the serum for random blood sugar and lipid profile tests. The biochemical tests were conducted using routine clinical assays and a colorimetric method, following the instructions provided by the automated system's manufacturer, utilizing the Accent-200 Analyzer, Cobas c111, and Cobas e411.

Primer design for quantification of real-time PCR (qPCR)

Primer has been designed in this study based on the Bioinformatics tools by using the international databases (NCBI) and several tools that are available on the website (online tools and software). The design process for primer was obtained by using primer3 plus and serial cloner 2-6-1 for all genes as appear in (Table 1 and Table 2).

RNA extraction and cDNA synthesis

Total RNA was extracted using the TransZol UP (TransGen Biotech, China) as specified in the manual for total RNA extraction. Using a NanoDrop spectrophotometer (NanoDrop Fisher, USA), the quantity and quality of the isolated RNA were determined. For RT-PCR, total RNA extracts were converted to cDNA according

Table 2: Primers used in the miRNA gene study.

Description	Sequence (5′→3′ direction)	Company and country
	***miRNA* gene**	
miRNA29-a	'5TAGCACCATCTGAAATCGGTTA'3	Alpha DNA company Canada
***miRU6* F.P.**	'5AGAGAAGATTAGCATGGCCCCT'3	
***miRNA*-universe R.P.**	'5-GCGAGCACAGATTAATACGAC'3	

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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

Quantitative real-time (qRT-PCR) for the *lncRNA H19* gene

To measure the threshold cycle (Ct), RT-PCR reaction mixtures were prepared with (Easy Script One-step gDNA Removal and cDNA Synthesis SuperMix) and performed on the Qiagen Rotor Gene Real-Time PCR System (Germany). Each reaction has been performed individually. Every reaction was carried out twice. The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase GAPDH was used as a reference gene. The gene expression reaction for each of GAPDH and H19 was performed separately under the following conditions: Enzyme activation at 95 °C for 1 min, denaturing at 95 °C for 15 sec, annealing temperature 64 °C for 30 sec, followed by 40 cycles, extension 72 °C for 15 sec and Melt curve from 60–95 °C, then fluorescence was measured.

Quantitative real-time (qRT-PCR) for the *miRNA29-a* gene

To measure the threshold cycle (Ct), RT-PCR reaction mixtures were prepared with (Easy Script One-step gDNA Removal and cDNA Synthesis SuperMix) and performed on the Qiagen Rotor Gene Real-Time PCR System (Germany). Each reaction has been performed individually. Every reaction was carried out twice. The housekeeping gene *miRNAU6* was used as a reference gene. The gene expression reaction for each of *miRNAU6*, as the housekeeping gene for *miRNA29-a*, was performed separately under the following conditions: Enzyme activation at 95 °C for 1 min, denature 95 °C for 15 sec, annealing temperature 64 °C for 30 sec, followed by 40 cycles, extension 72 °C for 15 sec and Melt curve from 60–95 °C then fluorescence was measured.

Statistical analysis

The data were presented as means±standard deviation (SD). The statistical analysis program used was SPSS 26.0 (SPSS Inc., Chicago, USA). Statistics were judged significant at p-value<0.05. A one-way ANOVA was used to statistically analyze the differences between the mean values of the control participants and the T2DM patients. Correlation analysis was conducted using the Pearson correlation test.

Results

The current study included measuring some biochemical tests of the T2DM patient groups and the healthy control groups. The results of random blood glucose showed that there was a highly significant difference between these two groups with a p-value (0.01), and the all-patients group had the highest difference from control (Table 3). The means of random blood glucose levels for the old patients and healthy controls were (201.41±76.941) and (103.66±16.925) mg/dl, respectively.

Quantification of *lncRNA H19* expression by real-time PCR

Table 4 presents the mean±SD Ct values of H19 cDNA amplification in patients' samples, specifically (22.35±1.939), in comparison to the corresponding Ct values in the control group (21.53±1.409), showing a highly significant difference (p≤0.01). The mean Ct values in the patient group were notably higher than those in the control group, suggesting a higher expression and up-regulation (fold 1.38) in the study groups (patients' group) as opposed to the control group (fold 1.00), as

Table 3: The biochemical test of the comparison of the T2DM patients' groups with the control group.

Parameters	Control Mean±SD	Patients Mean±SD	Standard error	P-value
RBS	103.66±16.925	201.41±76.941	16.971M 12.660 F	P<0.01**
HbA1C	5.31±0.704	8.63±8.912	0..21583 M 0.16146 F	P<0.001***
Total cholesterol	159.8±26.638	186.19±56.624	7.567 M 9.337 F	P<0.01**

Table 3: Continued.

Parameters	Control Mean±SD	Patients Mean±SD	Standard error	P-value
Triglyceride	143.78±35.410	173.36±70.771	11.288 M 12.598 F	P<0.01***
LDL	93.46±23.31	105.71±38.66	7.929 M 6.961 F	P<0.02**
HDL	51.88±7.926	44.08±12.450	2.787 M 2.490 F	P<0.01**
VLDL	28.25±7.83	34.36±13.679	2.003 M 2.926 F	P<0.05*
C.peptide	2.83±0.560	4.11±1.792	.362 M .375 F	P<0.03**

appears in Figure 1 AB). These results indicate an increased H19 gene expression in all patient groups, particularly suggesting that the H19 gene could serve as a diagnostic biomarker for type 2 diabetes mellitus.

The Ct value of *miRNAU6*, a housekeeping gene used in this work. The Ct values for *miRNAU6* among all study groups varied from 15–17, with mean±SD Ct values of (21.288±1.683) and (20.296±1.540) in patients and healthy groups. The Ct values of *miRNA29-a* (24.179±1.667) and (24.301±1.379) in patients and healthy groups, respectively, as appear in Figure 2 AB). These findings suggest a significant difference between the groups in terms of *miRNA-29a* Ct value means (p≤0.05). The analysis was performed in different study groups using the 2^-ΔCT value and the ratio of 2ΔCT of the different study groups (fold 2.17) to that of the control (fold 1.00) as appeared in Table 5.

A receiver operating characteristic (ROC) curve was generated for statistical analysis of H19 levels in these 100 samples. ROC is widely used to investigate a reasonable cutoff level of the target. The ROC curve displays the relationship between sensitivity and specificity for H19. ROC analysis of H19 revealed an area under the curve (AUC) of 0.86 (95% Confidence Interval (CI): 0.79, 0.93), p≤0.001. The ROC analysis revealed an optimal cutoff H19 value of 1.13, as appears in Figure 3. The optimal cutoff H19 value was less than or equal to 10 ng/µL, measured as absolute quantification by qPCR, and this value had a sensitivity of 68%, specificity of 96%, and a positive predictive value (PPV) of 81%.

A receiver operating characteristic (ROC) curve was generated for statistical analysis of *miRNA29-a* levels in these 100 samples. ROC is widely used to investigate a reasonable cutoff level of the target. The ROC curve displays the relationship between sensitivity and specificity for *miRNA29-a*. The ROC analysis revealed an optimal cutoff *miRNA29-a* value of predictive cutoff value (2.07) and the area under the curve (AUC) of 0.91 (95% Confidence Interval (CI): 0.84, 0.97), p≤0.001. The optimal cutoff *miRNA29-a* was less than or equal to 10 ng/µl measured as absolute quantification by qPCR, and this value had a sensitivity of 86% specificity of 92%, and a positive predictive value (PPV) of 98% as appeared in Figure 4.

The correlation between *lncRNA H19* gene and *miRNA29-a* gene with some parameters such as RBS and HbA1C

The results showed no significant relationship between H19 gene expression and RBS and HbA1C tests in the Iraqi patients with T2DM, with no significant

Table 4: Comparison of the lncRNA H19 gene in Ct, ΔCt values (Mean±SD) between investigation groups.

Group	Mean CT of H19 (mean±SD)	Mean CT of *GAPDH* (mean±SD)	ΔCT (mean Ct H19 -mean Ct GAPDH)	2-ΔCT	Experimental group/control group	Fold of gene expression	P-value
Patient	22.352±1.939	23.842±1.203	-1.49	2.81	2.81/2.04	1.38	P≤0.01
Control	21.53±1.409	22.555±1.394	-1.03	2.04	2.04/2.04	1.00	

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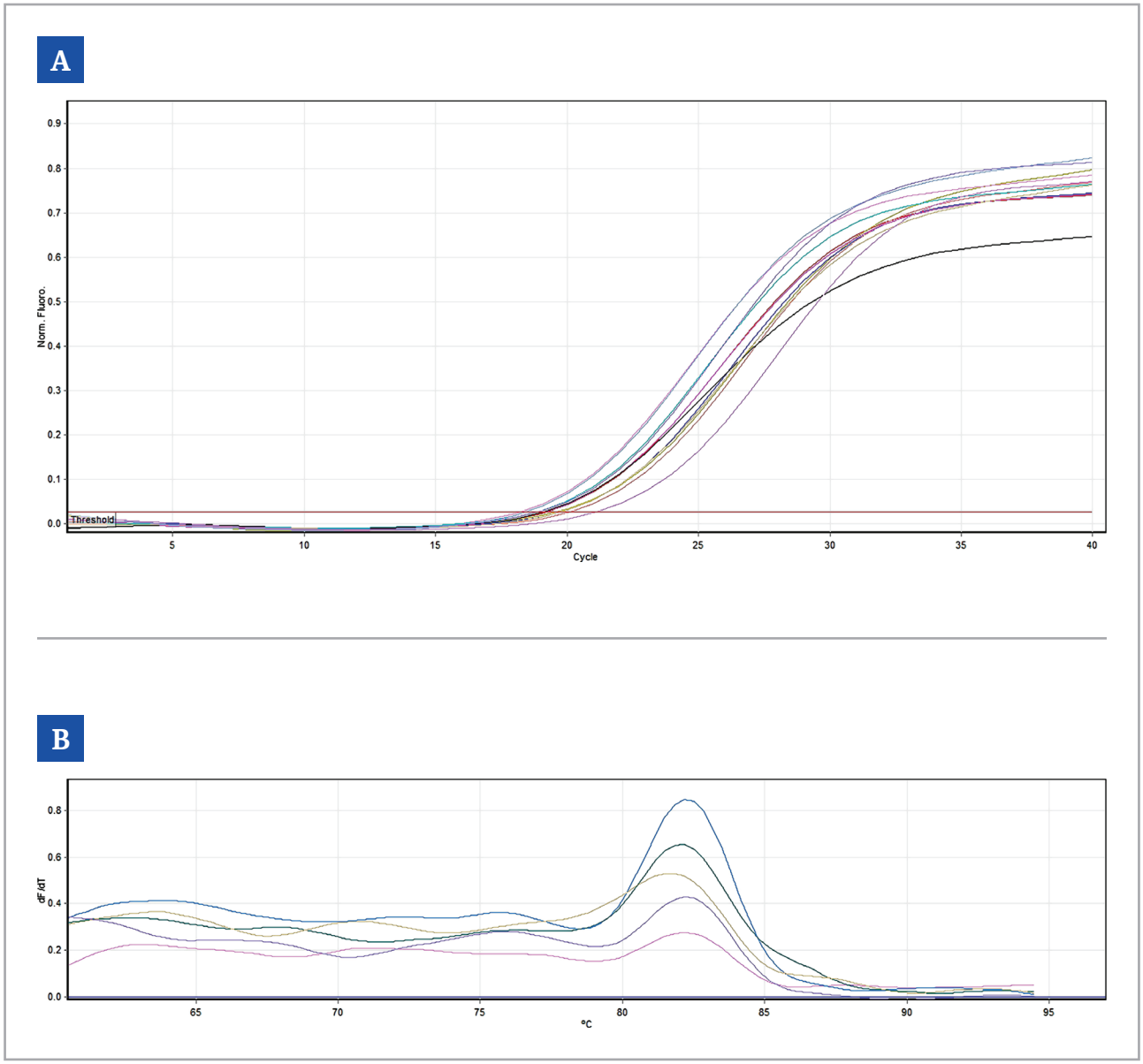

Figure 1: A – H19 gene amplification was plotted using qPCR samples that covered all research groups. The CT values varied between 15 and 17. B – H19 gene dissociation curves using qPCR samples that covered all research groups. Melting temperatures varied from 60°C to 95°C. The images were captured using the Qiagen Rotor Gene qPCR apparatus.

differences (P=0.990) and (p=0.747), respectively. The results showed no significant relationship between *miRNA-29a* gene expression and RBS and HbA1C tests in Iraqi patients with T2DM, with no significant differences (P=0.743) and (p=0.687), respectively.

The correlation between *miRNA29-a* gene and *lncRNA H19* gene

The results showed a positive correlation between the *miRNA-29a* gene and the *lncRNA H19* gene, as both genes exhibited increased gene expression (upregulation) with no significant differences (p=0.108).

Discussion

The results show that all parameters, including RBS, HbA1c, total cholesterol, triglycerides, LDL, HDL, VLDL, and C-peptide, are significantly higher in patients compared to the control group. This is due to several reasons, Type 2 diabetes (T2DM) is a metabolic disease that primarily affects adults and is brought on by inadequate or malfunctioning insulin, Diabetes mellitus type 2 Insulin resistance and reduced glucose tolerance, which results from the pancreas's insufficient capacity to secrete adequate insulin, are closely linked to type 2 diabetes mellitus (T2DM) and there is evidence

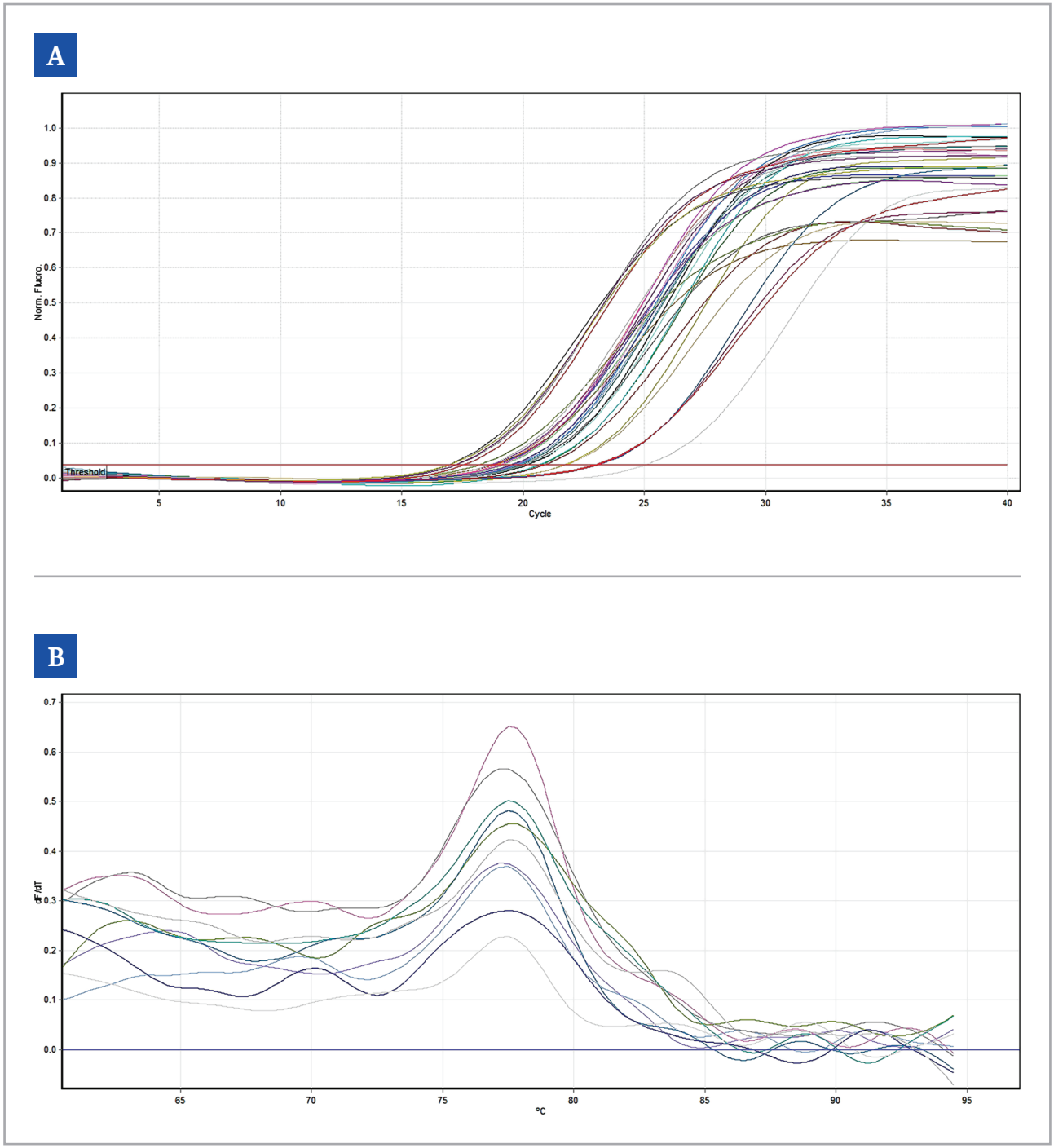

Figure 2: A – miRNA-29 a gene amplification was plotted using qPCR samples that covered all research groups. The CT values varied between 18 and 21.B – miRNA-29 a gene dissociation curves using qPCR samples that covered all research groups. Melting temperatures varied from 60°C to 95°C. The images were captured using the Qiagen Rotor Gene qPCR apparatus.

Table 5: Comparison of *miRNA29-a* gene and housekeeping gene *U6* in Ct, ΔCt values (Mean±SD) between investigation groups.

Group	Mean CT of *miRNA29-a* (mean±SD)	Mean CT of *U6*	ΔCT (mean Ct *miRNA29-a* -mean Ct *U6*)	2-ΔCT	Experimental group/control group	Fold of gene expression	P-value
Patient	24.179±1.6 67	21.288±1.6 83	2.891	0.13	0.13/0.06	2.17	P<0.05
Control	24.301±1.3 79	20.296±1.5 40	4.005	0.06	0.13/0.06	1.00	

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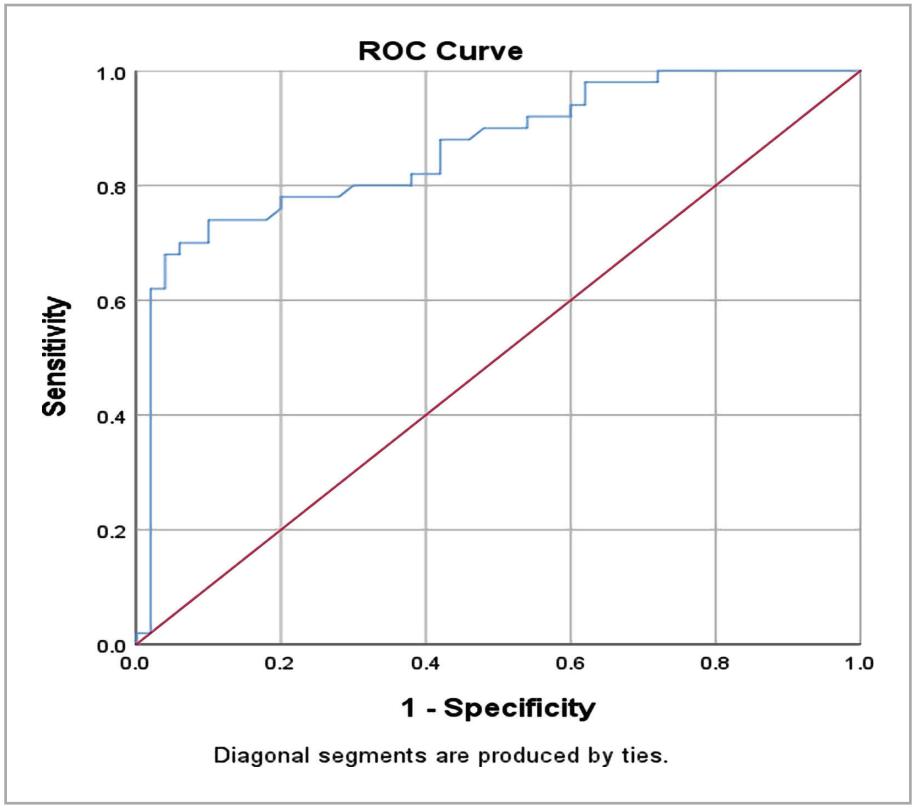

Figure 3: (ROC) curve was conducted on H19 level in plasm from non-diabetic (n=50) and diabetic patients (n=50) to establish the cutoff values. the area under curve (AUC) of 0.86 (95% CI: 79.3%, 92.6%) sensitivity of 68%, specificity of 96% the positive predictive value (PPV) of 84.6%.

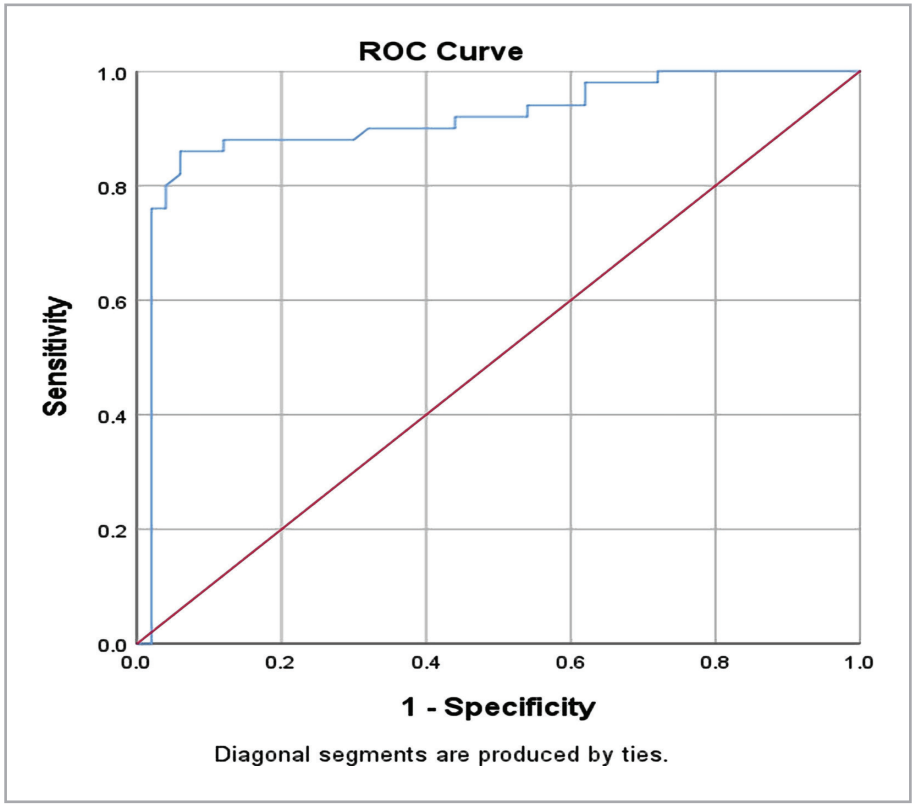

Figure 4: A receiver operating characteristic (ROC) curve was performed on *miRNA29-a* level in plasm from non-diabetic (n=50) and diabetic patients (n=50) to determine the cutoff values. area under curve (AUC) of 0.91 (95% CI: 72.3%, 92.6%) the sensitivity of 86%, specificity of 92% the positive predictive value (PPV) of 81%.

that VLDL-C overproduction is present in T2DM patients, and that this leads to increased levels of free fatty acids, hyperglycemia, obesity, and insulin resistance, also Because of their higher triglyceride levels, people with diabetes have smaller, denser LDL-C particles. Triglyceride lipolysis is compromised, or aberrant VLDL-C overproduction is the cause of elevated triglycerides. In the present study, the results show the means of random blood glucose levels for the old patients and healthy controls were (201.41±76.941) and (103.66±16.925) mg/dl, respectively. Regarding this result, a previous study by Patel *et al*. [11] and Dawood *et al*. [12] demonstrated that glucose concentrations in diabetic patients are significantly higher than in apparently healthy controls. The results showed that the mean average of glycated hemoglobin in the patient's group was (8.63±8.912), in comparison with the control group (5.31±0.704), respectively. These results are supported by Hussein and Saifalla [6], glycated hemoglobin (HbA1C) for all the studied patient groups, in addition to the control group (8.15±1.87) and (3.90±0.57), respectively.

A similar study, by Younus and Al-Faisal (2023) [13], stated that HbA1c is elevated in diabetics in the Iraqi population. The mean average of glycated hemoglobin was significantly increased (P>0.0001) from (5.3±0.704) in the serum specimen of the health controls group to (8.63±8.912) in the serum specimen of the T2DM patients' group [14]. The results of the cholesterol test showed the patient's group was (186.19±56.624), in comparison with the control group (159.8±26.638) mg/dl, respectively. In relation to this result, the mean cholesterol level in diabetic patients was significantly (p<0.0001) higher than that of the control group [12]. The results show that the mean serum triglyceride concentration in the T2DM group was (173.36±70.771), contrasting with (143.78±35.410) in the healthy control group. In a similar study described by Beshara *et al*. [15]. Demonstrates a significant association between increasing triglyceride levels, even within the accepted normal range, and the risk of development of type 2 DM. The results show that the mean value in diabetic patients (105.71±38.66) exhibited a statistically significant (p≤0.0001) increase compared to the control group's mean value (93.46±23.31). These results were supported by Dawood *et al*. [12]. The mean LDL-cholesterol value in diabetic patients was statistically significantly higher than that of the control group (p<0.0001). The results show a significant difference in the mean serum level of HDL between T2DM patients (44.08±12.45) and healthy controls (51.88±7.93), as reported by Hirano [16]. Who demonstrated that T2DM was usually associated with low plasma levels of HDL. In the current study, the results show (34.36±13.679) in T2D specimens compared to the mean level in healthy controls (28.25±7.83). In relevance to this result, a previous study by Dawood *et al*. [12]. The results showed that the mean level of VLDL significantly increased (P<0.0001), from (37.40±12.35) in the T2DM specimen in comparison with the mean level in healthy controls (19.32±4.14). The results show that the C-peptide level in the patient group was (4.11±1.792), while in the healthy group, it was (2.83±0.560). In a similar study, the C-peptide concentration was higher in T2DM (6.00±3.00) than in the non-diabetic group and Diabetic retinopathy (DR) in the Chinese population [17].

The results showed that the levels of circulating *lncRNA H19* and *miRNA-29a* expression were increased (upregulated) in T2DM patients compared to the control group. There are many dimensions that, in addition to genetic causes, include environmental factors, the type of drug, social factors, and differences in ethnic background. Several factors influence the expression of *miRNA-29a*, including exercise, lipid oxidation, obesity, insulin, and glucose uptake. MicroRNAs have emerged as important regulators of glucose and lipid metabolism in several tissues; however, their role in skeletal muscle remains poorly characterized. We determined the effects of the miR-29 family on glucose metabolism, lipid metabolism, and insulin responsiveness in skeletal muscle. They provide evidence that *miR-29a* and *miR-29c* are increased in skeletal muscle from patients with type 2 diabetes and are decreased following endurance training in healthy young men. The miR-29 acts as an important regulator of insulin-stimulated glucose metabolism and lipid oxidation, with relevance to human physiology and type 2 diabetes [18].

Recent reports indicate that relative H19 expression levels were significantly increased in the T2DM group compared to controls, while GAS5 levels were decreased in the T2DM group (p<0.001) [19] in the Egyptian population. In another study, it was found that serum levels of the *lncRNA H19* gene were decreased, while increased *miRNA-29a* was observed in patients with T2DM in the Saudi Arabian population [7].

The results show that ROC analysis of H19 revealed an area under the curve (AUC) of 0.86 (95% Confidence Interval (CI): 0.79, 0.93), p≤0.001. Moreover, the ROC analysis revealed an optimal cutoff *miRNA29-a* value of predictive cutoff value (2.07) and the area under the curve (AUC) of 0.91 (95% Confidence Interval (CI): 0.84, 0.97) p≤0.001. A similar study supported the use of a receiver operating characteristic (ROC) curve. The ex-

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pression levels of *miR-29a* and miR-147b show potential diagnostic performance in discriminating newly diagnoseddiagnosed T2DM (AUCs=0.77 and 0.84, respectively) [20].

The results showed no a significant relationship between *H19* gene expression with RBS and HbA1C tests in the Iraqi patients of T2DM with no significant differences (P-0.860) and (p-0.509) respectively these results are consistent with reports showing by Fawzy *et al.*, (2020) H19 and GAS5 expression profiles were not significantly correlated with clinical parameters including glycated hemoglobin (HbA1c) blood levels.

The results showed positive correlation between *miRNA29-a* gene and *lncRNA H19* gene because of both gene are increased gene expression (upregulation) with no significant differences ($p<0.769$). In similar study by Alfaifi etal., (2020) said that Spearman correlation was done to check the association of *lncRNA H19* with *miRNA-29a* Analysis showed a negative correlation of *lncRNA H19* expression with *miRNA-29a* ($r=-0.27$, $P<0.0001$).

Conclusion

The present study observed increased expression of cell-free *lncRNA H19*, as well as increased expression of *miRNA-29a*, in T2DM patients. The Relative *H19* expression levels were increased in the patient's group (fold 1.38) *vs*. controls (fold 1.00) with a highly significant difference ($p\leq0.01$), similar to *miRNA29-a* levels, which were also increased in the patient's group (fold 2.17) with a highly significant difference ($p<0.01$). The expression levels of lncRNA H19 and miRNA-29a are important in the diagnosis and pathogenesis of T2D.

Conflict of interest

The authors declare no conflict of interest.

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