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MicroRNA-499 gene expression in Egyptian type 2 diabetes mellitus patients with and without coronary heart disease

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Abstract: Type 2 diabetes mellitus (T2DM) is a cumulative lifelong disease with long term dangerous cardiovascular complications. MicroRNA-499 (miRNA-499) is a muscle-specific miRNA highly expressed in normal heart. This case control study was designed to assess serum levels of miRNA-499 gene expression in Egyptian T2DM patients in comparison with normal healthy volunteers and to explore its relationship with its coronary heart disease (CHD) complication. This study was performed on 180 individuals enrolled from Kasr Al-Ainy Hospital. They were divided into three groups; 60 T2DM patients with CHD, 60 T2DM patients without any complication and 60 healthy volunteers. Fasting blood glucose, HbA1c and lipid profile were assessed. Quantitative Real Time PCR was used for evaluation of miRNA-499 gene expression. Receiver Operating Characteristics (ROC) curve of miRNA-499 serum gene expression was performed to study the ability of miRNA-499 gene expression to discriminate between the studied groups. MiRNA-499 gene expression was downregulated in both diabetic groups ($p<0.001$**) than the control group and in diabetic group with CHD complication than diabetic group without complications ($p<0.05$**). ROC curve revealed that miRNA-499 gene expression discriminated between diabetic patients and controls with sensitivity 90% and specificity 96.6%. MiRNA-499 gene expression discriminated also between diabetic patients with and without CHD complication with sensitivity 73% and specificity 70%. MiRNA-499 downregulation was related to T2DM and its CHD complication risks. Collectively, miRNA-499 could be a beneficial biomarker for T2DM and its CHD complication and could discriminate between diabetic patients with and without CHD complication and normal individuals.

Keywords: Type 2 diabetes mellitus; Coronary heart disease; MiRNA-499; MiRNA-499 gene expression.

1. INTRODUCTION

Type 2 diabetes mellitus is a metabolic disturbance identified by hyperglycemia due to insulin resistance particularly in liver, skeletal muscle, and adipose tissue that represent a great bluster to worldwide health of individuals because of its strong connection to atherosclerotic vascular disease with significant long-term morbidity and mortality[1,2]. Oxidative stress is a key component in the development and progression of T2DM and its vascular complications such as CHD[3,4]. The plaque formation in T2DM patients causes narrowing of the coronary arteries and hence heart attacks[5].

Despite significant advancements in prehospital emergency treatment and in-hospital reperfusion therapy over the past two decades, CHD is the main single reason of death globally. More than 70% of deaths in diabetics are due to heart disease or stroke[6]. A large ratio of this burden is found in low and middle income countries. This accounts for nearly 7 million deaths annually and is a huge global economic burden[7].

Hence, it is of great importance to search for reliable and novel molecular biomarkers that are capable of predicting the onset and the progress of T2DM and could even be targeted for treatment. MicroRNAs are fundamental for gene expression regulation in several critical cellular processes such as cellular proliferation, cell cycle and apoptosis[8]. In tumorgenesis and their aberrations microRNAs were also found to have significant roles in cancer genesis and progression. Therefore, they are good candidates for many diseases diagnosis and therapy[8-10]. MicroRNAs regulate gene expression through

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cleavage or inhibition of translation of target mRNAs during or after transcription[11].

This work is a part of a study done on Egyptian patients suffering from type 2 diabetes mellitus with and without CHD to investigate miRNA-499 gene expression level in these groups compared to normal healthy volunteers and also to study the association of miRNA-499 rs3746444 A>G genotype variants with type 2 diabetes mellitus and its coronary heart disease complication.

The present manuscript focuses on the first part of the study which is the relation of miRNA-499 gene expression level with the studied groups, we assumed that miRNA-499 might have a role in the pathogenesis of CHD on top of T2DM as it is encoded by the myosin heavy chain 7B (MYH7B) gene located in the heart[12,13]. Moreover, miRNA-499 expression level in normal heart is high while in different heart diseases was found to be downregulated[14].

2. METHODS

2.1. Study population

This study is a case control study that was performed on one hundred and eighty individuals. One hundred and twenty T2DM patients were recruited from the outpatient clinic of Kasr Al Ainy Hospital. Patients were diagnosed clinically then categorized into two groups; sixty T2DM patients without any complication and sixty T2DM patients with CHD. Diabetes mellitus was defined as a fasting blood glucose (FBG) $\geq$ 126 mg/dl (7.0 mmol/L) or HbA1c $\geq$ 6.5% (48 mmol/mol)[15]. Sixty healthy volunteers (sex and age matched with the patients' groups) were enrolled as a control group. A written approval was taken from all participants. The study was approved by the ethics committee of the Faculty of Pharmacy (Girls), Al-Azhar University (REC no. 254). Patients suffering from type 1 diabetes mellitus, any type of cancer, liver and kidney diseases, any metabolic disorders that may affect the studied biochemical parameters or any other diabetic complications than CHD were excluded.

2.2. Samples collection

Ten ml of blood were withdrawn from every participant under complete aseptic conditions after an overnight fasting (12 hours) and divided into three portions: Two ml were collected in fluoride coated tube for determination of fasting blood glucose (FBG), Three ml were collected in EDTA tube and mixed gently for glycated hemoglobin assay (HbA1c) on fresh whole blood and three ml were left at room temperature to clot, centrifuged for 15 minutes at 3000 rpm then serum was detached, divided into aliquots and kept at -80^{o}c until assessment of the lipid profile, liver and kidney function tests, and miRNA-499 gene expression.

2.3. Quantitative real-time polymerase chain reaction for serum gene expression of miRNA-499

MicroRNA was extracted from serum samples using mirvana kit USA (catalog no. AM1560). The isolated miRNA was reverse transcribed into cDNA using specific miRNA primers for miRNA assay and reagents (TaqMan® MicroRNA Reverse Transcription Kit (catalog no. 4366596). Each 15µL RT reaction consisted of 7µL master mix, 3µL primer and 5µL RNA sample with performing the following thermal condition; the temperature was held at 16°C for 30 minutes, then at 42°C for 30 minutes and finally at 85 °C for 5 minutes. Amplification of cDNA was carried out on step-one real- time PCR system by AmpliTaq Gold DNA polymerase (catalog no. N8080240).

Analysis of data was done by relative quantification using $2^{-\Delta\Delta ct}$ method. In this method, data was offered as the fold change in microRNA expression normalized to an endogenous control and relative to the healthy groups.

ΔCT = CT (miRNA-499) - CT (endogenous control)

$\Delta\Delta CT$= ΔCT (patient) - ΔCT (healthy groups)

Fold Change or (Rq) = $2^{-\Delta\Delta CT}$

Table 1. Sequences of the primers used for real time PCR:

Genes	Primers Sequence	Gene bank accession number
MiRNA-499	Forward primer: 5'-CAA AGT CTT CAC TTC CCT GCC A-3' Reverse primer: 5'-GAT GTT TAA CTC CTC TCC ACG TGA TC-3'	NR_039912.1
Housekeeping miRNA; RU6	"Forward primer: 5-GGAACGATACAGAGAAGATTAGC-3" "Reverse primer: 5-TGGAACGCTTCACGAATTTGCG-3"	XR_005231505.1

2.4. Statistical Analysis

Statistical Package for Social Science (SPSS) version 26 was used for data entry and statistical analysis. Qualitative data were represented by frequencies and percentages while quantitative data were represented by mean, median and standard deviation (SD) to measure central tendency and dispersion of quantitative data. Chi square (X^2) test was used for the comparison between qualitative data, Odds ratio (OR) with 95% confidence intervals were calculated. Independent t-test was used for the comparison between two quantitative data and ANOVA for the comparison between more than two quantitative data. Ranked spearman correlation was

used to assess the association between serum expression levels of miRNA-499 and some risk factors. Level of significance was taken at *p*-value <0.05. The Receiver Operating Characteristics (ROC) curve was constructed using MedCalc version 14 to obtain the most sensitive and specific cutoff values that could discriminate between groups, area under the curve (AUC) have been also calculated.

3. RESULTS

3.1. Baseline characteristics of the study population

All the studied individuals were matched in the baseline characters (age, sex and BMI). There was a highly significant increase in FBG level of both diabetic groups when compared to the control group (*P*<0.001). Also, upon comparing the HbA1c% of both diabetic groups with controls, there was a highly significant increase in the former groups (*P*<0.001) (figures 1, 2). Consistently, there was an extremely significant increase in triglycerides, total cholesterol and LDL-C on comparing both diabetic groups with the control group (*P*<0.001) but, there was a highly significant decrease in HDL-C on comparing both diabetic groups with the control group (*P*<0.001) (figure 3).

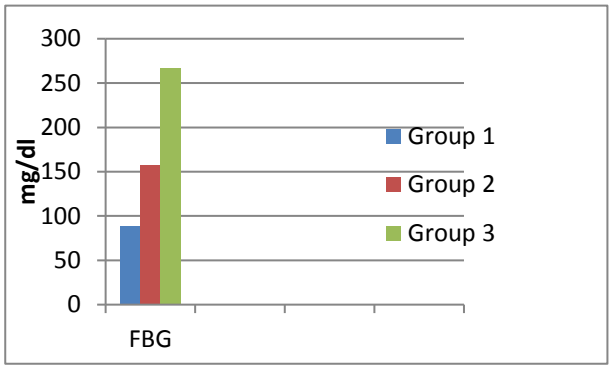

Figure 1: Fasting blood glucose of the studied groups.

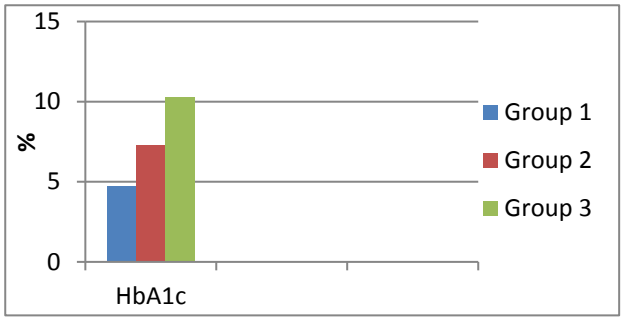

Figure 2: HbA1c of the studied groups.

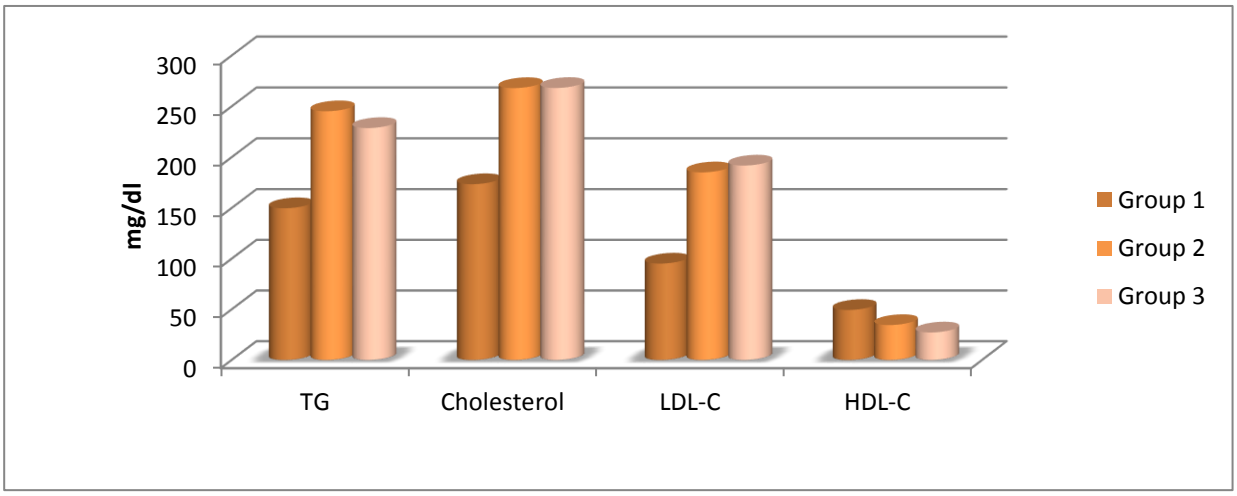

Figure 3: Lipid profile of the studied groups.

3.2. miRNA-499 gene expression level of the study population

Regarding miRNA-499 gene expression, there was a highly significant reduction in its serum gene expression level in both diabetic groups compared to the control group (*P*<0.001) and a significant lowered gene expression level in type 2 diabetic patients with CHD than type 2 diabetic patients without complications (*P*<0.05) (Table 2).

3.3. Receiver Operating Characteristics (ROC) curve of miRNA-499

Receiver Operating Characteristics (ROC) curve of serum gene expression level of miRNA-499 for discrimination between diabetic patients and controls revealed an AUC of 0.97 with sensitivity 90% and specificity 96.6% (*P*<0.0001). ROC curve of serum gene expression level of miRNA-499 for discrimination between diabetic patients with and without CHD complication revealed an AUC of 0.72 with sensitivity 73% and specificity 70% (*P*<0.0001) (Tables 3, 4) (figures 4, 5).

Table 2: Median of serum miRNA-499 gene expression level of the studied groups.

Groups / Parameters	Controls	Diabetic Patients		*p*-value
	Group 1 N=60	Group 2 N=60	Group 3 N=60	
RQ miRNA-499				
Median	1.18	0.479[a]	0.327[a,b]	$p<0.001^{**}$
(1st quartile- 3rd quartile)	(0.96-1.5)	(0.34-0.69)	(0.2-0.46)	

Group 1: Control group; Group 2: T2DM patients without complications; Group 3: T2DM patients with CHD.
a: significance from group 1
b: significance from group 2
* Significant at $p\leq0.05$, ** highly significant at $p\leq0.001$.

Table 3: ROC curve of serum miRNA-499 gene expression for discrimination between diabetic patients and controls.

AUC	Cutoff point	%Sensitivity	%Specificity	*p*-value
0.97	0.70	90	96.6	$p<0.0001$

Table (4): ROC curve of serum miRNA-499 gene expression for prediction of CHD complication in diabetic patients.

AUC	Cutoff point	%Sensitivity	%Specificity	*p*-value
0.72	0.40	73	70	$p<0.0001$

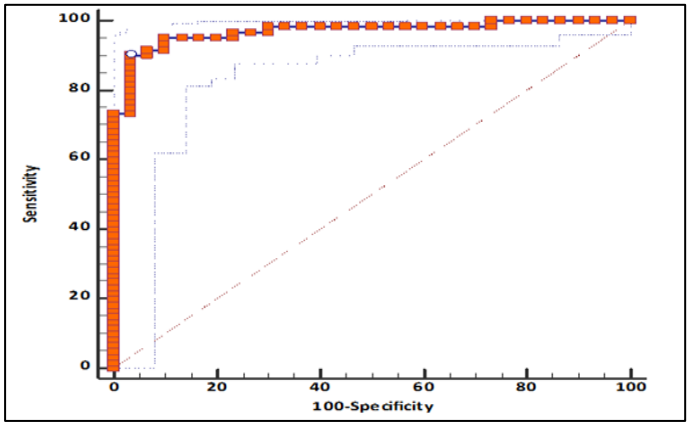

Figure 4. ROC curve of serum miRNA-499 gene expression for discrimination between diabetic patients and controls.

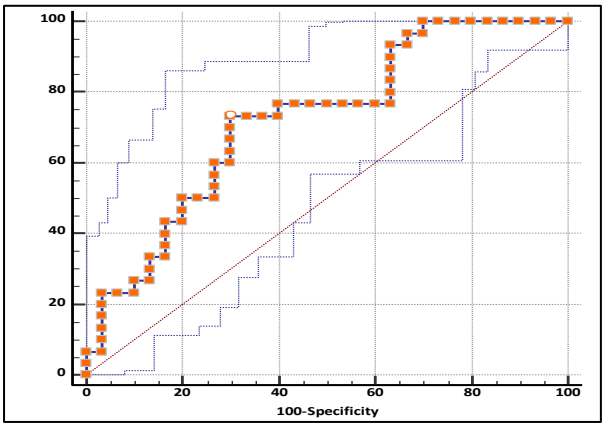

Figure 5. ROC curve of serum miRNA-499 gene expression for prediction of CHD complication in diabetic patients.

3.4. Correlation of miRNA-499 gene expression with the studied parameters among all the studied diabetic patients:

A significant negative correlation was obtained between miRNA-499 gene expression and each of FBG (mg/dl) and HbA1c (%) among the studied diabetic patients ($p<0.001$) (table 5).

4. DISCUSSION

Type 2 diabetes mellitus is among the most rapidly growing global health emergencies[16]. It has been the main cause of death in the developed world for many years[17].

Hyperglycemia, insulin resistance and hyperinsulinemia in T2DM accelerate atherosclerosis as they are associated with more atherogenic dyslipidemia which stimulates leukocyte and platelet sticking together, clotting, inflammation and coronary plaque ulceration[18].

MicroRNAs play vital roles in various biological processes under physiological conditions or pathological disorders as they are involved in metabolism, development, immune response, tumorigenesis, metastasis, diabetes mellitus, cardiovascular diseases, apoptosis and autophagy [19,20]. Recently, miRNAs were proven to be promising candidate biomarkers in cardiovascular diseases as they are noninvasive parameters that can be easily detected in body fluids (blood, urine, etc.) due to their remarkable stability and presence in apoptotic bodies and exosomes[21].

In the current study, all groups were matched in the baseline characters avoiding selection bias. Fasting blood glucose and glycated hemoglobin were significantly highly increased in diabetic patients' groups in comparison with the control group and significantly highly increased in the diabetic group with CHD complication than without complications ($p<0.001$) (figures 1, 2). These results were in

harmony with the criteria reported by the American Diabetes Association for the diagnosis of DM which states that a diabetic patient has either FBG <126 mg/dl, or HbA1c < 6.5%[22]. Besides, glycated hemoglobin test is the most common diagnostic and screening tool used for T2DM monitoring and research and is considered the gold standard of diabetic care in contemporary clinical practice [23]. In the present study, both diabetic groups showed significantly higher TG, Cholesterol and LDL-C levels and significantly lowered HDL-C level as compared to the control group (figure 3). However, no significant differences were obtained between both diabetic groups regarding lipid profile; this could be explained by the hypo-cholesterolemic drugs taken by diabetic patients with CHD.

Table 5: Correlation of miRNA-499 gene expression with the studied parameters among all studied diabetic patients.

Parameters	MiRNA-499 gene expression level in the studied diabetic patients n= 120	
	r-value	*p*- value
Age (years)	-0.181	0.048*
Weight (kg)	-0.029	0.756
Height (m)	-0.141	0.124
BMI (Kg/m^2)	0.083	0.368
ALT (U/L)	0.101	0.271
AST (U/L)	0.193	0.305
Urea (mmol/L)	-0.003	0.972
Creatinine (mg/dl)	0.11	0.23
FBG (mg/dl)	-0.346	<0.001**
HbA1c (%)	-0.404	<0.001**
TG (mg/dl)	0.113	0.219
Total Cholesterol (mg/dl)	0.009	0.921
HDL-C (mg/dl)	0.066	0.476
LDL- C (mg/dl)	-0.014	0.876

* Significant at $p \leq 0.05$, ** highly significant at $p \leq 0.001$.

These results came in accordance with Mellor et al., who defined type 2 diabetes mellitus as a condition of hyperglycemia associated with suppressed HDL cholesterol[24]. Also, Al Mahmeed et al., reported that hyperlipidemia was widely spread in patients with CHD[25].

This case control study was designed to evaluate miRNA-499 serum gene expression levels in Egyptian T2DM patients with and without CHD compared to normal healthy volunteers.

In previous studies, miRNA-499 was found to be expressed in normal heart and circulating miRNA-499 was suggested to be a sensitive biomarker for acute myocardial infarction[13]. It was also found to protect cardio-myocytes from H_2O_2 induced apoptosis[26].

MicroRNAs have been proved to be implicated in the regulation of the pancreatic β-cells function, insulin-signaling pathways, glucose stimulated insulin secretion, autophagy/apoptosis interplay and other biological behaviors related to glucose metabolism disorders making them ideal candidates for unearthing T2DM molecular complexities[27,28]. This comes in line with the present study as serum gene expression of miRNA-499 in both T2DM groups revealed significantly lowered levels than that in the control group. Moreover, its values were significantly lowered in T2DM with CHD than those without complications (p<0.001) (table, 2). These results were agreed with Mohsen et al., who stated that hyperglycemia induces miRNA-499 down-regulation and explained their results with increased oxidative stress. They added that miRNA-499 down-regulation mediated diabetic cardiomyopathy[29]. Also, Chavali et al., reported down regulation of miRNA-499 in diabetic hearts, suggesting miRNA-499 as a putative candidate implicated in the pathophysiology of diabetic heart disease[30]. Yildirim et al., reported that miRNA-499 down-regulation have mediated diabetic cardiomyopathy and suggested that hyperglycemia induced miRNA-499 down regulation[31].

Hathaway et al., found a link between extreme ROS production in STZ-induced diabetic cardiomyopathy and decreased expression of miRNA-499[32]. Huang et al., explained that in db/db mice's livers, the downregulated miRNA-499-5p diminished the glycogen synthesis and insulin signaling pathways[33]. Moreover**,** Nigi et al., stated that miRNA-499-5p was decreased in pancreatic islets from insulin resistant rodent models via directing apoptosis-associated tyrosine kinase (AATK). They found miRNA-499-5p could

compensate β-cell mass expansion. Moreover, they demonstrated that miRNA-499-5p directly targets PTEN phosphatase[34].

It was simply expected that endothelial activation associated with coronary artery disease (CAD) would induce the liberation of micro particles and remnants of apoptotic cells, thereby elevating the miRNAs levels. However, Zernecke et al. explained that miRNA can reach the atherosclerotic lesion via apoptotic bodies. Therefore, they speculated that the reduction in circulating miRNAs seen in patients with CHD might be caused by the uptake of freely movable miRNAs from the circulation into the atherosclerotic lesions[35].

In order to show the discriminative power of miRNA-499 between control group and diabetic groups, Receiver Operating Characteristics (ROC) curve was carried out and showed specificity 96.6% and sensitivity 90% at cut off value 0.70 and AUC 0.97. Also, ROC curve for discrimination between both diabetic patients' groups (with and without cardiac complications) showed specificity 70% and sensitivity 73% at cut off value 0.40 with AUC 0.72, tables (3,4), figures (4, 5). Hence, miRNA-499 could be considered as a beneficial diagnostic biomarker for discrimination between diabetic patients and normal individuals and also between diabetic patients with and without CHD complication.

These results were agreed with previous researches that studied the relation of miRNA-499 with cardiac diseases, such as a study on Egyptian population by Fawzy et al., who reported that circulating miRNA-499a could function as a useful biomarker in discriminating acute MI from healthy volunteers within 12 hours[36] and another two studies by Mayr et al., and Zhao et al., who reported that miRNA-499 was a worthy diagnostic and/or prognostic marker across different cardiovascular disease progression stages[37,13]. Also, Wang et al., reported that miRNA-499 had a high diagnostic efficiency for prediction of acute coronary syndrome (ACS)[38].

In the current study, the correlation between miRNA-499 gene expression, and clinical data among the studied DM patients (with and without CHD) revealed a negative significant correlation between miRNA-499 gene expression and each of age, FBG and HbA1c (table 5). The decline in the levels of miRNA-499 with age was expected as proceeding in age is well known to be accompanied with an increase in the incidence and prevalence of diabetes mellitus and its CHD complication.[39, 40] Furthermore, in agreement with our results, Fluitt et al., found a negative significant correlation between miRNA-499 gene expression and HbA1c in African American pre-diabetic adults. They considered miRNA-499 a hopeful biomarker for early DM diagnosis, and added that miRNA-499 targeted PTEN and was involved in both insulin and glucose tolerance[41].

5. CONCLUSIONS

According to the studied Egyptian population, miRNA-499 seemed to exert a protective activity against T2DM and its CHD complication. Its downregulation could play a role in the pathogenesis of both. Also, miRNA-499 could be a beneficial biomarker for discrimination between diabetic patients with and without CHD complication and normal individuals.

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Conflicts of Interest: The authors report no conflict of interest.

Ethical Statement: The present work was approved by the Research Ethical Committee of the Faculty of Pharmacy (Girls), Al-Azhar University, Cairo, Egypt (REC number: 254).

Author Contribution: This work was carried out in collaboration between all authors. HEO: methodology, formal analysis and writing the manuscript. NAE: data analysis, visualization, validation, editing manuscript, and gaining ethical approval. SSE: supervision, data analysis and editing the manuscript. LAR: the practical part and data curation. All authors have read and approved the final manuscript.

List of Abbreviations: T2DM: Type 2 diabetes mellitus, miRNA-499: microRNA-499, CHD: Coronary heart disease, ROC: Receiver operating characteristics, FBG: Fasting blood glucose, HbA1c: Glycated hemoglobin, MYH7B: Myosin heavy chain 7B, AUC: Area under the curve, SPSS: Statistical package for social science, SD: Standard deviation, CAD: Coronary Artery Disease, AATK: Apoptosis-associated tyrosine kinase, ACS: Acute coronary syndrome, mRNA: Messenger RNA.

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