MICRO RNA192 EVALUATION AS EARLY DIABETIC RETINOPATHY DIAGNOSTIC BIOMARKER IN Egyptian PATIENTS WITH TYPE 2 diabetes mellitus

FATH ELBAB AMANY G.[1], M. Y. NASR[1], GHADA M. I. NASR[2] AND M. I. NASR[1]

1. Molecular Biology Department, Genetic Engineering and Biotechnology Research Institute (GEBRI), University of Sadat City, Egypt
2. Molecular Diagnostics Department, Genetic Engineering and Biotechnology Research Institute (GEBRI), University of Sadat City,Egypt

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Diabetes is a major and quickly spreading health issue on a global scale. One of the most prevalent metabolic illnesses in the world is Type 2 Diabetes Mellitus (T2DM), which is primarily brought on by the interaction of two key factors: impaired insulin production by pancreatic beta-cells and impaired insulin sensitivity in tissues (Roden and Shulman, 2019). About 90-95% of all instances of diabetes worldwide are T2DM, and this number is continually rising (Hegazi *et al*., 2015). With approximately 8,850,400 cases and an adult prevalence of 15.2%, Egypt ranks ninth globally (Azzam *et al*., 2021). Microvascular problems including retinopathy, nephropathy, and neuropathy as well as macrovascular consequences are all highly correlated with T2DM (An *et al*., 2021).

The most prevalent microvascular consequence of diabetes and the main factor contributing to blindness globally is diabetic retinopathy DR (Ting *et al.*, 2016). The World Health Organization estimates that between 1980 and 2014, the incidence of diabetes increased by around 29%, and that the frequency of diabetes-related early mortality is increasing (NCD-RisC, 2016). Due to the increased prevalence of diabetes globally, DR become the major cause of blindness in people of working age. DR has an impact on patients personally, but it also places a significant financial and healthcare cost on society (WHO, 2021).

A major class of short (22 nt) non-coding RNAs called microRNAs works to inhibit the translation of messenger RNA targets and/or hinder protein synthesis. The target messenger RNA's 3′-UTR (untranslated) region contains complementary sequences to bind (O'Brien *et al.*, 2018). Numerous critical procedures pertaining to cellular development, apoptosis, differentiation, metabolism, and immune

response are controlled by these short RNAs (Annese *et al*., (2020). MicroRNAs (miRNAs) have a role in the microvascularization associated with DR, and miRNAs whose expression changes during the pathogenesis of DR have been reported (Mastropasqua *et al*., 2014). Similar to this, certain miRNAs regulate the pathophysiology of DR by acting on a variety of targets, including the immune system, fibrosis, oxidative stress, inflammation, and cell function, in response to different signaling pathways. The phenotypes of serum miRNAs may develop into novel types of diagnostic indicators (Deshpande *et al*., 2018; Wang *et al*., 2019). MiR-192 is one of the earliest studied miRs that controls pathogenic pathways triggered in DR, however its impact on DR is still debatable

This study's goal was to evaluate miR-192 expression and determine its potential as blood-based biomarkers in patients with T2D who were developing diabetic retinopathy and diabetic nephropathy.

SUBJECTS AND METHODS

Study design and population

Hundred patients who attended the Internal medicine Clinic and the Diabetes Specialized Clinic at El Menoufia University Hospital were the subjects of a case study. Four groups of people were created: a healthy non-diabetic control group of 30 person, 35 diabetic patients without complications, and 35 diabetic patients and diabetic retinopathy. The study excluded participants who had a history of chronic diseases. Patients, who were being treated for diabetes using diet, oral anti-diabetic drugs, and/or insulin to achieve glycemic control, as well as those with fasting plasma glucose levels ≥ 126 mg/dl and haemoglobinA1c levels ≥ 6.5 %, were also included in this study. The participants' age, sex, fasting blood glucose (FBG) levels, glycated haemoglobin (HbA1c), serum creatinine, lipid profile, as well as alanine liver functions, and complete blood count (CBC), were all examined.

Blood collection and microRNA isolation

Participants provided peripheral blood samples (5 ml), which were drawn from them into EDTA-coated tubes for RNA isolation. Observing the guidelines provided by the manufacturer, whole blood was used to isolate total RNA that contained small RNA using the MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche, Germany, Cat. no. 03730964001). RNA concentration and quality were evaluated with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc.). By comparing the absorbance ratios of 260/280 nm and 260/230 nm, RNA purity was ascertained. The final concentrations of each RNA sample were identically diluted: 20 ng/µl.

Quantification of miR-192 expression level

Observing the guidelines provided by the manufacturer, 100 ng of miR was reverse transcribed into complementary DNA (cDNA) using the Reverse Tran-

scription Kit (Thermo Scientific) and stem-loop primers unique to miRNA. With the Real-time 7500 Fast PCR System and Applied Biosystems' SensiSMARTTM SYBR Master Mix, the quantitative Real-Time (qRT-PCR) analysis was carried out twice (Thermo Fisher Scientific). Each reaction had a final volume of 20 µl and contained a cDNA template, SensiSMARTTM SYBR Master Mix, and nuclease-free water. The Applied Biosystems Application Note recommended using the non-coding short RNA U6 snRNA (internal control). The relative expression levels of the target miRNAs were demonstrated using the difference in Ct between the target miRNAs and U6 snRNA (ΔCt), which is comparable to the ratio of log2-transformed absolute copy numbers. Predenaturation at 95ºC for 2 minutes, followed by 40 cycles of 95ºC for l0 seconds of denaturation and 60ºC for 1 minute of annealing and extension, were the prescribed reaction conditions set in accordance with the manufacturer's protocol. The difference between the cycle threshold (CT) value of miRNA-192 and the average CT value of reference genes across all samples in a particular sample set serves as the expression for this miRNA.

Statistical Analysis

Utilizing SPSS (Statistical Package for Social Sciences) version 25 for Windows®, the gathered data were coded, processed, and analyzed (IBM SPSS Inc, Chicago, IL, USA). Frequency distributions and relative percentages were used to display qualitative data. To compare between two or more sets of qualitative variables, the chi-square test (χ^2) was used. The mean SD format was used to express quantitative data (Standard deviation). To compare between two independent groups of normally distributed variables, the independent samples t-test was utilized (parametric data). Two-tailed P values were used to determine statistical significance ($p<0.05$). In order to calculate the diagnostic indices (sensitivity, specificity, positive and negative predictive values, and accuracy) for micro-RNA 192, the Receiver Operating Curve (ROC) test was employed to distinguish between the diseased (diabetic retinopathy) and un-diseased (control) groups.

RESULTS

Demographic and clinical data of study participants

Table (1) summarizes the demographic and biochemical data for both patients and healthy controls. The current study involved 70 T2DM patients—38 males and 32 females-as well as 30 healthy people, of whom 16 were men and 14 were women. Patients with T2DM were divided into two groups: those without ocular problems, consisting of 35 individuals (22 male and 13 female), and those with DR, consisting of 35 patients (24 males and 11 females). Regarding age and sex distribution, there were no statistically significant variations between the two groups. Diabetes patients had significantly higher levels of the biochemical

markers FBG, HbA1c, total cholesterol, LDL, HDL, and triglycerides, as well as ALT and AST, than healthy controls ($P< 0.05$).

Blood relative expression of miR-192 and diabetic complications

The expression levels of miR-192 in the blood of diabetic patients and healthy non-diabetic controls were assessed using qRT-PCR analysis. The largest value was in patients with DR with a significant difference. The relative expression of miR-192 in diabetic patients' blood indicated a direct link with diabetes complications. The expression level of miR-192 shown in Table (2) is explained by data. In terms of DR severity, the level considerably rises as the disease progresses. Table (3) displayed the correlation between biochemical variables and the levels of mir-192 expression in each group under investigation.

After ROC analysis (Fig. 1), the area under the curve (AUC) for miR-192 was 0.967 (95% confidence interval [CI], CI0.790 - 1.000) with DR. A cutoff value of >0.68 was chosen from a range of ROC analysis cutoff values, as the sensitivity of 83.3% and specificity of 100% at the selected cutoff were optimal for miR-192 with DR (Table 4).

DISCUSSION

It is acknowledged that type 2 diabetes is a serious public health problem that has a significant impact on human life and healthcare costs. In many regions of the world, rapid economic growth and urbanization have led to an increase in the prevalence of diabetes (Onyango and Onyango, 2018). The majority of people with T2DM have at least one complication, such as DR, which are the leading causes of morbidity and mortality (Zheng *et al*., 2017). The ability of traditional diabetes indicators like FBG and HbA1c to predict the likelihood of acquiring diabetic complications in a sensitive group is limited. MiRNAs have the ability to be more effective problem-specific indicators associated with diabetes. Current treatment approaches for diabetes management worldwide need for the discovery of distinctive miRNA profiles to identify diabetes and, ideally, to determine the likelihood of acquiring diabetes-related problems in a vulnerable population (Banerjee *et al*., 2017).

In this study, there was no statistically significant difference in age or gender distribution across the analyzed groups. This is in line with the findings of Saadi *et al*., (2019), who observed no significant changes in gender or age distribution across all study groups. In terms of biochemical analysis, FBG, HbA1c, Cholesterol, Triglyceride, LDL ($P<0.001$), HDL ($P<0.05$), and ALT, AST ($P<0.001$) all increased statistically significantly in the current study. This is in line with Rai and Rai (2018), who found that T2DM without complications and T2DM with nephropathy had significantly higher TC, TG, LDL-c, and HbA1c values when compared to controls. T2DM

without complications and T2DM with nephropathy had significantly lower HDL-c levels when compared to controls.

The focus of this study was to confirm if miRNA-192 expression level variations are implicated in diabetes microvascular complications and know if there is a correlation between miRNA-192 expressions and diabetic retinopathy, with the purpose of diagnosis. Compared to the control group, all diabetic groups had significantly higher mean expression levels of circulating miRNA-192, according to the study's findings ($P< 0.0001$). These results are in line with those of Khamis *et al*. (2021), who discovered that neutrophil gelatinase-associated lipocalin (NGAL) and miRNA-192 levels were significantly higher in T2DM patients. Hamdia *et al*. (2013) demonstrated that diabetics have blood miR-192 levels that are significantly higher than non-diabetics, with levels even higher in patients with long-term disease without microvascular problems. In contrast to Ma *et al*., (2016) and Lotfy *et al*., (2021), who found a statistically significant decrease in micro RNA-192 levels in macro-albuminuria compared to other groups, as well as in microalbuminuria compared to normal albuminuria and healthy control.

In the present study, there was a significant positive association between the levels of miRNA 192 expression and the diabetic retinopathy group's blood sugar, HbA1c, and cholesterol ($P<0.05$), but not with triglycerides, HDL cholesterol, ALT, or AST ($P> 0.05$). Creatinine showed a negative connection ($P<0.05$). While in keeping with the same study's findings regarding creatinine, Yang *et al*. (2017) discovered that the expressions of serum miR-192 were adversely linked with HbA1c.

In this study with DR, the area under the curve (AUC) of miR192 was 0.967 (95 % confidence interval [CI], CI0.790 - 1.000). A cutoff value of >0.68 was chosen because miR-192 had a sensitivity of 83.3 % and a specificity of 100 % at the chosen cutoff, As a result of these findings, it was shown that detecting miR192 lowered the incidence of false positives in diabetic retinopathy patients.

CONCLUSION

According to the findings, up-regulated expression of miRNA-192 in type 2 diabetes is a risk factor for the progression of renal and ocular complications in diabetics. MiRNA-192 may act as early markers of changes in particular biological processes in the retina, as well as molecular signatures in diabetic microvascular complications. In clinical practice, the miR192 cutoff values were crucial. The diagnostic, prognostic, therapeutic, and use of anti-miRNA-192 in different diabetic microvascular problems all need further investigation. We can increase the sample size, follow the cases, and go more deeply into the underlying mechanism in the future

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ABSTRACT

Background and Objective: Short non-coding RNAs known as microRNAs have been associated with different disorder types, like diabetes mellitus (DM) and its complications such diabetic retinopathy and nephropathy. In order to ealy diagnose diabetic retinopathy (DR), the study purpose was to assess the expression level of miRNA 192 in type 2 diabetes patients and explore its association with these problems.

Subjects and Method: The participants in the current study were 30 healthy non-diabetic people and 70 type 2 diabetes patients who were categorized into two main groups according to the time from the onset of DM (age and sex-matched). Diabetic retinopathy is one of the most common consequences of diabetes. The complete set of data was collected, including sociodemographic and laboratory data. RT-PCR assay was used to determine the levels of miRNA192 expression in whole blood.

Results: All diabetic groups, particularly diabetic patients with retinopathy, had mean expression levels of miRNA 192 that were considerably greater than those of healthy subjects. The expression levels of miRNA 192, blood glucose and HbA1c, were significantly positively correlated in the group with diabetic retinopathy. Mir-192 had a sensitivity of 83.3% in diabetic retinopathy and specificity of 100 % at the specified cut-off.

Conclusion: According to the findings, up-regulated of miRNA 192 in type 2 diabetes is correlated to the prevalence of diabetic retinopathy. Warning indications of diabetes complications could be miRNA 192.

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Table (1): Data on the demographics and biochemistry of the all study population.

Variables		Control (n=30)	Diabetic (n =35)	DR (n=35)	P-value
Gender: M/F		16/14	22/13	24/11	P>0.05
Age		45.67 ± 5.99	48.03 ± 4.71	46.71 ± 5.17	P>0.05
Duration of T2DM		--	5.3±1.21	5.9± 3.56	P>0.05
FBG(mg/dl)		79.4±4.7	142.5±15.1	157.3±19.8	P<0.001
HbA1C (%)		4.9±0.5	7.6±0.4	8.2±0.6	P<0.001
Cholesterol(mg/dl)		167.2±21.4	189.6±22.8	229.5±24.6	P<0.001
Triglyceride(mg/dl)		132.5±18.8	191.1±28.3	249.3±37.6	P<0.001
HDL cholesterol(mg/dl)		51.4±2.3	48.6±3.5	46.9±3.4	P>0.05
LDL cholesterol(mg/dl)		103±19.1	107±18.5	128±28.5	P<0.001
ALT(IU/L)		18.39±7.42	28.8± 7.2	30.4± 6.9	P<0.0001
AST(IU/L)		20.29±8.24	29.3 ± 7.9	32.6± 7.7	P<0.0001
Creatinine (mg/dl)		0.77±0.17	0.79±0.18	0.74±0.13	P>0.05
CBC	Hb	11.6±2.3	11.2±2.6	10.8±3.1	P>0.05
	TLC	6.6±3.9	6.9±3.8	7.1±4.1	P>0.05
	PLT	223±52.9	214±54	205±49	P>0.05

P value< 0.05 is significant

Table (2): Comparison of the expression levels of miRNA 192 in all studied groups.

Groups	miRNA192 mean± SD	*P* value
Non-diabetic healthy Control	0.40± 0.23459	P < 0.0001
T2DM	2.568± 0.539	
Diabetic retinopathy (DR)	4.624± 1.33	

Table (3): Correlations between mir-192 expression levels with biochemical Parameters in patient groups.

mir-192 expression level	T2DM (n = 30)		DR (n = 35)	
	r	P	r	P
FBG(mg/dl)	-0.66	<0.05	0.70	<0.05
HbA1c (%)	-0.54	<0.05	0.74	<0.05
Cholesterol (mg/dl)	1	<0.05	0.99	<0.05
Triglyceride (mg/dl)	-0.38	>0.05	0.98	>0.05
HDL cholesterol (mg/dl)	-0.46	>0.05	0.95	>0.05
ALT(IU/L)	-0.5	>0.05	0.48	>0.05
AST(IU/L)	-0.5	>0.05	0.44	>0.05
Creatinine	- 0.26	<0.05	-0.38	<0.05

r: Pearson correlation significance at P<0.05

Table (4): Validity of micro RNA-192 in the diagnosis of DR.

Parameters	Cutoff value	AUC	95% CI	Sensitivity	Specificity	PPV	NPV	P-value
DR	>0.68	0.967	0.790 -1.000	83.3%	100%	100%	85.7%	<0.001

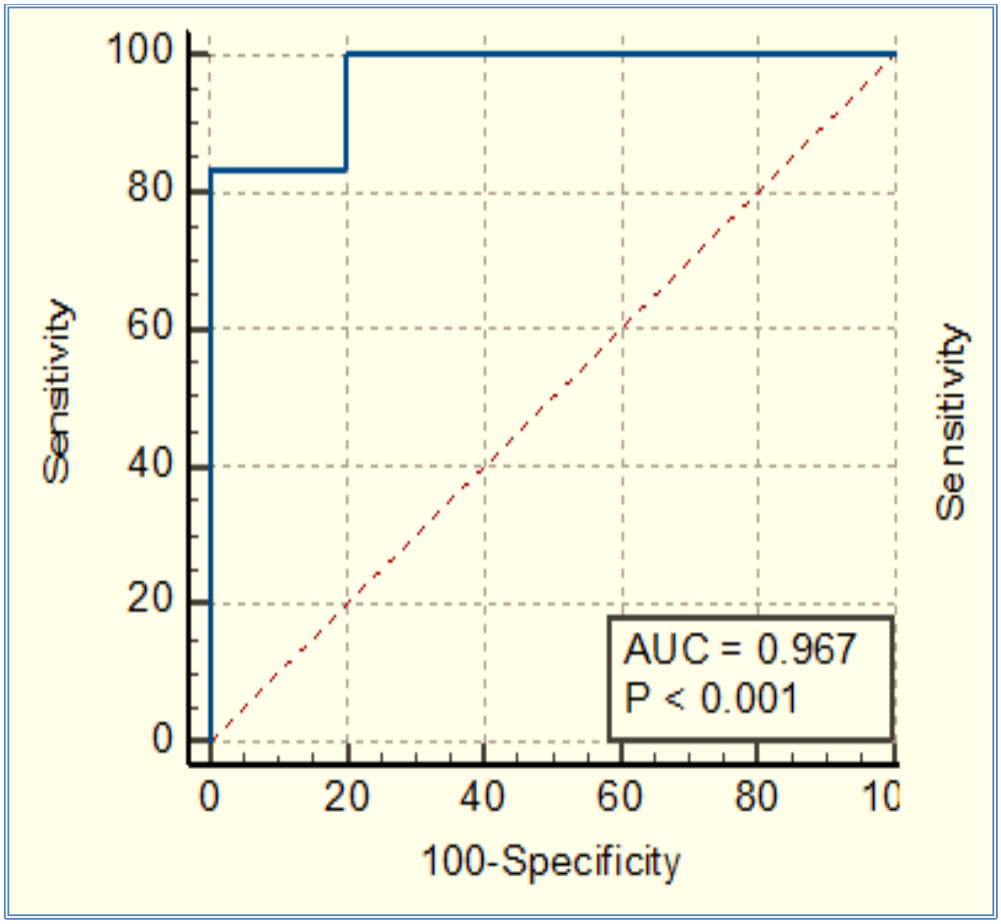

Fig. (1): The ROC curve of miR-192 in DR group.