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MICRORNA-224 UP-REGULATION: A RISK FOR COMPLICATIONS IN TYPE 2 DIABETES MELLITUS EGYPTIAN PATIENTS

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ABSTRACT

Type 2 diabetes mellitus (T2DM) is a major public health concern facing the world today. Recent studies suggested that the expression of biomolecules including microRNAs especially changes during the progression of T2DM, and its related complications, and is suggestive of disease pathology. This study aimed to investigate miRNA-224 expression among T2DM Egyptian patients in addition to evaluating its relationship to diabetic complications. A total of 205 individuals were subjected to this case-control study, cases were allocated to 100 T2DM patients and 105 healthy ones. The biochemical, anthropometric, psychometric parameters and miRNA-224 expression by Real-time PCR technique were measured. T2DM patients had significantly elevated levels of glycated hemoglobin (HbA1C), fasting blood suger (FBS), post prandial blood sugar (PPBS), international normalization ratio (INR), creatinine, urea, cholesterol, triglyceride (TG) and low density lipoproteins (LDL) and significantly decreased high density lipoprotein (HDL) compared to healthy subjects. MiRNA-224 expression was significantly upregulated in T2DM patients compared to healthy individuals p (<0.001), MiRNA-224 showed superior diagnostic potential for diabetes and predicted diabetic risk among non-diabetic groups. Diabetic complications including retinopathy, neurological, eye complications, joint pain and cardiac disease were more frequent in T2DM patients than in control subjects. These differences were statistically significant at *p* (<0.05). Diabetic complications were more frequent with high expression of miRNA-224 >1.065. In conclusion, microRNA-224 could be considered as a novel diabetes-associated miRNA which is highly expressed in the serum of T2DM patients. Also it is highly expressed in patients who have diabetic complications at *p* (<0.05).

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Introduction

Diabetes Mellitus type 2 (T2DM) is manifested by a lack of insulin action, secretion, or both, which leads to hyperglycemia. The prevalence of diabetes, particularly T2DM, has dramatically increased worldwide, and it is estimated by 2040, to reach up to 629 million people [1]. T2DM is the most prevalent kind of disease, particularly in developing countries [2]. The prevalence in Egypt is around 15.6 % of all adults aged 20-79.

Long-term hyperglycemia is thought to be the cause of the harm done to many organs, increasing morbidity and mortality. Poor glycemic control is the primary cause of endothelial dysfunction, which in turn affects several intracellular pathways linked to both micro and macrovascular problems [3]. The principal microvascular consequences of diabetes are nephropathy, neuropathy, retinopathy, cerebrovascular, and cardiac diseases. About 25% of cases with T2DM have diabetic nephropathy and retinopathy [4]. The capillary basal lamina, which includes glomerular arterioles, heart, retina, muscle, and skin, is impacted by poor glycemic control and thickens, which results in the development of microvascular problems [5].

Also, it is considered a risk factor for the development of these problems including hyperlipidemia, genetic susceptibility, advanced glycation end products, hypertension, growth factors, and inflammatory mediators in addition to hyperglycemia [6].

In the last twenty years, our understanding of the biological regulation of genetic material was greatly developed [7]. The genome is containing 2% protein-coding genes and more than 90% non-coding protein genes. The protein-coding genes are transcribed to RNA and then translated to protein. Noncoding genes are transcribed to non-coding RNA but not translated to proteins [8]. Non-coding RNA has a very important role in genetic regulation. MiRNAs are non-coding RNA that was shown to have a significant role in different diseases [9]. It is composed of ~22 nucleotides [10].

miRNA had a potential role in the posttranscriptional gene expression regulation by one of two mechanisms; the first is suppression of mRNA translation and the second is the degradation of mRNA. More than 60% of the protein-coding genes have miRNA target sites in their 3'-UTR. Therefore, those genes are under miRNA regulation [11].

A novel degree of gene regulation in glucose equilibrium called miRNAs has evolved in addition to normal insulin secretion and action to regulate glucose normal levels in the body. [12] revealed that in pancreatic endocrine cell lines there were specific miRNAs were upregulated. Besides their functions in insulin synthesis and secretion, miRNAs also regulate insulin signaling in tissues that insulin targets [13].

Additionally, different diabetic problems are greatly influenced by epigenetic patterns including histone changes, DNA methylation, and non-coding RNAs, particularly miRNAs [14]. miRNAs are crucial for cell division, cellular differentiation, proliferation, and death. Previous research on miRNAs demonstrated the significance of miRNA in the SARS-CoV2 infection [15]. The additional study highlighted the significance of miRNAs like miR-155 and miR-665 as potential non-invasive hepatocellular carcinoma indicators in chronic hepatitis C virus-infected Egyptian cases [16].

Short non-coding RNAs (miRNAs) had a significant effect on the gene expression post-transcriptional control by playing a role in the silencing of particular mRNAs. The majority of miRNAs are generated from particular DNA sequences as pri-miRNAs, which are then transformed into pre-miRNA and mature miRNAs. The majority of miRNAs interact with the 3′-UTR of their target mRNAs, leading to mRNA breakdown and translation suppression a result. Many studies found that miRNAs are associated with other areas, such as 5′-UTR and promoter coding sequences [4].

The imbalance of miRNAs has been connected to various biological activities, such as cell division, proliferation, apoptosis, and development. Excellent predictors of diabetes and its consequences are miRNAs. Specific miRNAs have an impact on cellular viability, insulin secretion, resistance, and macro- and microvascular problems. Previous research has shown that particular miRNAs are disrupted in vascular, renal, and retinal cells. In vivo models of this diabetes, consequences have also shown this. The importance of miRNAs in the development and/or prevention of numerous diabetes problems is because they specifically target the key genes implicated in these complications [6].

The importance of this miRNA as a biomarker of these complications has also been examined in this study, along with its value as a prognostic indicator and therapeutic target Retinopathy and ocular issues are two common consequences.

Materials and Methods

Subjects

A total of 205 participants, including 100 people with type 2 diabetes and 105 healthy controls, were enrolled in this case-control study through the National Institute of Diabetes and Endocrinology's Outpatient clinic. This study was reviewed and approved by the ethical committee of GOTHI (General Organization of Teaching Hospital and Institution) (NO: IDE00288)

The diagnosis of T2DM cases was according to the American Diabetes Association (2020) guidelines (a fasting plasma glucose level ≥ 7.0 mmol or 126 mg/dl) [17]. The Diabetic patients who met the eligibility requirements and experienced problems like (retinopathy, cardiac diseases, and neurological complications) were included in the study. Every participant was questioned about risk factors and demographic information. Each participant has his anthropometric and physiological measurements taken, including waist circumference (WC), pulse pressure (PP), systolic blood pressure (SBP), diastolic blood pressure (DBP), height, and weight. Each included case was asked to provide written informed consent. The participant, who was barefoot and wearing light clothing, had his or her anthropometric measurements (height and weight) obtained. Individuals' weight and height were calculated to the closest 0.1 kg and 0.5 cm using standardized methodologies. The weight (in kg) divided by height (in m) was assessed for body mass index (BMI) evaluation. WC was evaluated using conventional

anthropometric methods to the closest 0.1 cm. After a minimum of 5–10 minutes of rest, SBP and DBP were assessed three times, and the average of the three values was used.

Biochemical Analysis
After fasting for 12 h overnight, 15 ml of venous blood was collected and plasma was collected to evaluate postprandial blood glucose, high-density lipoproteins (HDL-C), triglycerides (TG), total cholesterol (TC), and random blood sugar by using Olympus AU 400(Automated biochemistry analyzer). Values for LDL-C were evaluated by Firewall's [18].

Blood Collection and Storage
15-milliliter blood samples were taken and divided among several vacutainers. The blood was placed in yellow gel vacutainers, which underwent centrifugation at 4000 rpm for 10 minutes after 30 minutes to separate the sera from the clot-like whole blood. Two aliquots of sera were created, with the first aliquot being used for RNA extraction. Up until use, every aliquot was kept frozen at 80 ºC.

Assay of Serum miRNA-224
Qiagen miRNeasy Mini kit (Qiagen, Hilden, Germany) was used for total RNA extraction from serum samples as directed by the manufacturer. Using a NanoDrop 1000 spectrophotometer, the amount of RNA was measured spectrophotometrically (NanoDrop Tech, Wilmington, DE). By using qRT-PCR, the expression of MicroRNA-224 was measured. A TaqMan® microRNA reverse transcriptase kit (Applied Biosystems Cat. No. 4366596) and the particular miRNA primers have miR-224 5x was used to perform reverse transcription (RT) (Applied Biosystems, Cat. No. 4427975). Internal control was implemented using RNU6B. Prior to usage, for maintaining a constant concentration of total RNA in the RT reaction, sera were diluted in RNase-free water. RT reactions (15 µL) contained 7 µL of master mix, 3 µL primer, and 5 µL (1 – 10 ng) of diluted RNA. RT was done in a Master cycler Gradient at 16°C for 30 min., 42°C for 30 min, 85°C for 5 min, then held at 4°C. TaqMan® Universal Master Mix 40R (Applied Biosystems Cat. No. 4440043). Real-time PCR was done by MX 3000 Applied Biosystems.

Statistical Analysis
Statistical Analysis Software for the Social Sciences was used to process the data (SPSS, version 24). Median, range, mean, and standard deviation were used to convey numerical data, whereas numbers and percentages were used to represent qualitative data. Whenever necessary, the chi-square (Fisher's exact) test was performed to analyze the correlation between the qualitative variables. The odds ratio and its 95% confidence interval were calculated for multivariate analysis using a Logistic regression model to test for the independent predictive influence of univariately significant factors. A ROC (Receiver operating characteristics) analysis and a logistic regression model were used to compute the sensitivity, specificity, positive predictive value, negative predictive value, and overall accuracy, along with their respective 95% confidence intervals. The cutoff for statistical significance was set at a p-value of less than or equal to 0.05. Every test was two-tailed.

Results and Discussion

The study involved 205 participants. This comprised 100 T2DM cases, 61 (61%) were males and 39 (39.0%) were females, and 105 healthy controls, with 61 (58.1%) males and 44 (41.9%) females. Among T2DM cases, the number of patients <=median value was 52 (52%) and the number of patients > median value was 48 (48%). whereas, among the control group, the number of patients <=median value was 53 (50.5%) and the number of patients > median value was 52 (49.5%) **(Table 1)**. There was no significant difference between diabetic and control groups regarding gender, age, smoking, and BMI with p-values of (0.672, 0.372, 0.581, and 0.827, respectively).

Table 1. Comparison between studied groups regarding demographic variables

		Group		p-value
		control (n=105)	**diabetic group (n=100)**	
Gender	Male	61 (58.1%)	61 (61.0%)	0.672
	Female	44 (41.9%)	39 (39.0%)	
Age (years)	<=median value	53 (50.5%)	52 (52.0%)	0.827
	> median value	52 (49.5%)	48 (48.0%)	
Smoking	No	84 (80.0%)	83 (83.0%)	0.581
	Yes	21 (20.%)	17 (17.0%)	
BMI	<=25	31 (29.5%)	24 (24.0%)	0.372
	>25	74 (70.5%)	76 (76.0%)	

Positive significant correlations were found among participants regarding WBC, HCT%, MCHC, Neutrophil, HbA1C %, FBS, PP, INR, Creatinine, Urea, TG, and LDL **(Table 2)** between the control and diabetic group.

Table 2. Comparison between studied groups regarding laboratory findings

		Group		P value
		control (n=105)	**diabetic group (n=100)**	
Hb (g/dl)	Normal	45 (42.9%)	39 (39.0%)	0.575
	Abnormal	60 (57.1%)	61 (61.0%)	
RBC (Million/cmm)	Normal	41 (39.0%)	38 (38.0%)	0.878
	Abnormal	64 (61.0%)	62 (62.0%)	
WBC	Normal	72 (68.6%)	0 (0.0%)	<0.001
	Abnormal	33 (31.4%)	100(100.0%)	
HCT%	Normal	73 (69.5%)	30 (30.0%)	<0.001
	Abnormal	32 (30.5%)	70 (70.0%)	
MCH (pg)	Normal	76 (72.4%)	80 (80.0%)	0.201
	Abnormal	29 (27.6%)	20 (20.0%)	
MCHC (g/dl)	Normal	31 (29.5%)	56 (56.0%)	<0.001
	Abnormal	74 (70.5%)	44 (44.0%)	
Neutrophil (g/L)	Normal	84 (80.0%)	2 (2.0%)	<0.001
	Abnormal	21 (20.0%)	98 (98.0%)	
Lymphocyte (g/L)	Normal	82 (78.1%)	86 (86.0%)	0.141
	Abnormal	23 (21.9%)	14 (14.0%)	
Monocyte (g/L)	Normal	78 (74.3%)	63 (63.0%)	0.081
	Abnormal	27 (25.7%)	37 (37.0%)	
HbA1C %	<5.7	$105_{a\ (100.0\%)}$	$20_{\ (20.0\%)}$	<0.001
	>5.7-<6.4	$0_{a\ (0.0\%)}$	$12_{b\ (12.0\%)}$	
	>6.4	$0_{a\ (0.0\%)}$	$68_{b\ (68.0\%)}$	
FBS (mg/dl)	Normal	63 (60.0%)	0 (0.0%)	<0.001
	Abnormal	42 (40.0%)	100(100.0%)	
PPBS (mg/dl)	Normal	97 (92.4%)	1 (1.0%)	<0.001
	abnormal	8 (7.6%)	99 (99.0%)	
Creatinine	Normal	105(100.0%)	94 (94.0%)	0.012
	abnormal	0 (0.0%)	6 (6.0%)	
Urea	Normal	103 (98.1%)	91 (91.0%)	0.024
	abnormal	2 (1.9%)	9 (9.0%)	
Cholesterol	Normal	103 (98.1%)	79 (79.0%)	<0.001
	abnormal	2 (1.9%)	21 (21.0%)	
TG	Normal	97 (92.4%)	82 (82.0%)	0.026
	abnormal	8 (7.6%)	18 (18.0%)	
HDL	Normal	72 (68.6%)	17 (17.0%)	<0.001
	abnormal	33 (31.4%)	83 (83.0%)	
LDL (U/L)	Normal	0 (0.0%)	33 (33.0%)	<0.001
	abnormal	105(100.0%)	67 (67.0%)	

Moreover, there were statistically marked variations in the groups regarding retinopathy with p values <0.001 in **(Table 3)**. Also, there was a considerable association in the case of neurological complications.

Table 3. Comparison between studied groups regarding diabetic compilations.

		Group		p-value
		control (n=105)	**diabetic group (n=100)**	
Retinopathy	No	104 (99.0%)	83 (83.0%)	<0.001

	Yes	1 (1.0%)	17 (17.0%)	
eye complication	No	105(100.0%)	74 (74.0%)	<0.001
	Yes	0 (0.0%)	26 (26.0%)	
neurological complication	No	105(100.0%)	91 (91.0%)	0.001
	Yes	0 (0.0%)	9 (9.0%)	
cardiac diseases	no	105(100.0%)	97 (97.0%)	*
	yes	0 (0.0%)	3 (3.0%)	

The Receiver Operating Characteristic (ROC) curve was plotted using the sensitivity and specificity values derived for each micro-RNA 224 level detected in Type 2 Diabetes Mellitus research sample **(Figure 1 and Table 4)**.

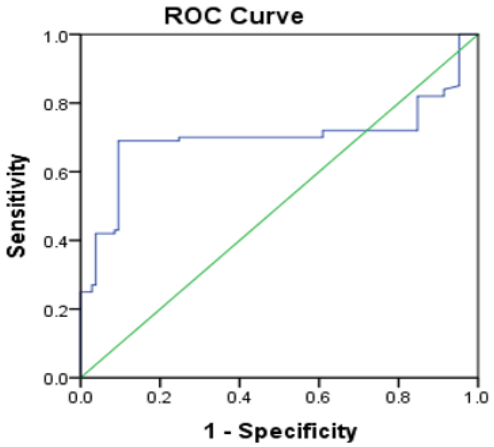

Figure 1. ROC curve displayed from the sensitivity and specificity values determined for each micro-RNA 224 level measured in the study sample, with Type 2 Diabetes Mellitus.

Table 4. miRNA 224 cut-off point.

	Sensitivity	Specificity	PPV	NPV	AUC	P value	Total Accuracy	SE	CI	
MiRNA224	69.0%	90.5%	87.3%	75.4%	0.698	< .001	80	0.041	Lower 0.617	Upper 0.779

The Association of microRNA-224 expression in the studied population correlated with clinical characteristics, and laboratory data showed that HCT% (p = 0.001), MCHC (p= 0.002), urea (p = 0.017), cholesterol (p = 0.001), and retinopathy (p =0.010) were also found to have significant correlation with microRNA-224 expression.
The relationship of microRNA-224 expression in the studied population was correlated with clinical characteristics, and laboratory data and is summarized in **(Table 5)**.

Table 5. Serum microRNA-224 expression in the studied population correlated with laboratory findings

		microRNA-224		p-value
		<=1.065	**>1.065**	
WBC	Normal	64 (50.8%)	8 (10.1%)	<0.001
	Abnormal	62 (49.2%)	71 (89.9%)	
HCT%	Normal	75 (59.5%)	28 (35.4%)	0.001
	Abnormal	51 (40.5%)	51 (64.6%)	
MCH (pg)	Normal	95 (75.4%)	61 (77.2%)	0.766
	Abnormal	31 (24.6%)	18 (22.8%)	
MCHC (g/dl)	Normal	43 (34.1%)	44 (55.7%)	0.002
HbA1C %	<5.7	100 (79.4%)	25 (31.6%)	<0.001
	>5.7-<6.4	3 (2.4%)	9 (11.4%)	
	>6.4	23 (18.3%)	45 (57.0%)	
FBS (mg/dl)	Normal	54 (42.9%)	9 (11.4%)	<0.001
	Abnormal	72 (57.1%)	70 (88.6%)	

PP (mg/dl)	Normal	87 (69.0%)	11 (13.9%)	<0.001
	Abnormal	39 (31.0%)	68 (86.1%)	
INR	Normal	70 (55.6%)	15 (19.0%)	<0.001
	Abnormal	56 (44.4%)	64 (81.0%)	
Creatinine	Normal	124 (89.4%)	75 (94.9%)	0.208
	Abnormal	2 (1.6%)	4 (5.1%)	
Urea	Normal	123 (97.6%)	71 (89.9%)	0.017
	Abnormal	3 (2.4%)	8 (10.1%)	
Cholesterol	Normal	119 (94.4%)	63 (79.7%)	0.001
	Abnormal	7 (5.6%)	16 (20.3%)	
TG	Normal	114 (90.5%)	65 (82.3%)	0.086
	Abnormal	12 (9.5%)	14 (17.7%)	
HDL	Normal	72 (57.1%)	17 (21.5%)	<0.001
	Abnormal	54 (42.9%)	62 (78.5%)	
LDL (U/L)	Normal	10 (7.9%)	23 (29.1%)	<0.001
	Abnormal	116 (92.1%)	56 (70.9%)	
Retinopathy	No	120 (95.2%)	67 (84.8%)	0.010
	Yes	6 (4.8%)	12 (15.2%)	
eye complication	No	120 (95.2%)	59 (74.7%)	<0.001
	Yes	6 (4.8%)	20 (25.3%)	
neurological complication	No	123 (97.6%)	73 (92.4%)	0.076
	Yes	3 (2.4%)	6 (7.6%)	
joint pain	No	119 (94.4%)	74 (93.7%)	0.818
	Yes	7 (5.6%)	5 (6.3%)	
Waist	<=median value	68 (54.0%)	41 (51.9%)	0.773
	>median value	58 (46.0%)	38 (48.1%)	
cardiac diseases	No	125 (99.2%)	77 (97.5%)	*
	Yes	1 (0.8%)	2 (2.5%)	

MicroRNA-224 expression was also observed to have a highly significant correlation ($p < 0.001$) to PPBS **(Figure 2)**.

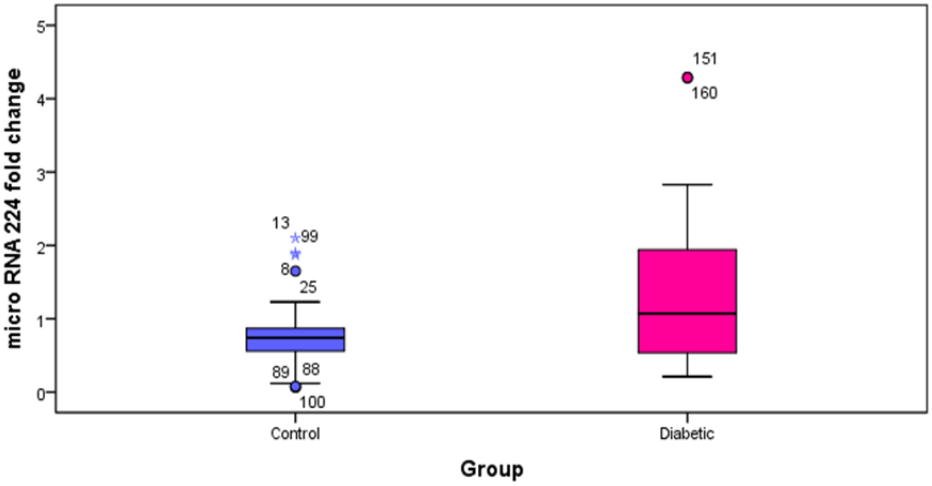

Figure 2. Comparison between the studied groups regarding microRNA-224 expression.

The association of complicated and uncomplicated DM in the studied patients was correlated with microRNA-224 expression, clinical characteristics, and laboratory data and is described in **Table 5**. No significant correlation was found with microRNA-224 expression (P=0.480). A remarkable relationship was observed between complicated and uncomplicated diabetic patients

with Hb (p =0.024) and RBC (p = 0.015). MicroRNA-224 expression was directly proportional to control and diabetic patients **(Figure 2)**.

The study involved a total of 205 participants. In 100 cases with T2DM subjects, 61% of them were males and 39.0% were females, and 105 control, with 58.1% males and 41.9% females. Therefore, the predominance of the participants was males, in contrast to a similar study [19] in which the majority of the participants were females. There were no marked variations between control and diabetic groups regarding gender, age, smoking, and BMI. This is in line with another study without a remarkable relationship between smoking and BMI on diabetes risk [20]. However, another study found that having a higher BMI was correlated with a greater risk of diabetes [21].

Positive significant correlations were found among the participants regarding HbA1C %, FBS, PPBS, TG, Cholesterol, HDL, and LDL. This is consistent with another study which found a remarkable positive association between HbA1c, FBS, cholesterol, LDL-C, TG, and LD [22]. There were statistically significant positive correlations among the participants regarding WBC, HCT%, MCHC, and Neutrophil. This is matched with another research in which remarkable variations ($p<0.05$) between diabetic and healthy children regarding hemoglobin, red blood corpuscles (RBCs) count, hematocrit, white blood cells (WBCs) count, mean platelet volume, and MCH values [23].

There were statistically significant positive correlations in the participants regarding Creatinine, and Urea. This is similar to a study that reported a marked positive relationship between FBS and uric acid plasma levels, blood parameters, urea, and creatinine [24]. Moreover, there were statistically obvious variations in the studied groups regarding retinopathy, with p values < 0.001. A significant positive relationship was found between the duration of diabetes and the grade of retinopathy ($P<0.01$), glycosylated hemoglobin ($P<0.01$), and fasting blood glucose ($P<0.01$) [25]. Also, there was a significant correlation in the case of neurological complications (p = 0.001). Increased HbA1c levels were found to be a significant predictor of polyneuropathy in diabetic patients [26].

This is agreed with a recent study, which reported that microRNA-224 is easily detectable in the urine of diabetic cases and may be a signal of β-cell destruction. Clinical and biochemical variables were shown to be associated with expression levels [27]. This shows that these linked parameters (HbA1C and MicroRNA expression), when combined, can effectively identify diabetic individuals from healthy individuals and can be utilized as diabetes predictors. Furthermore, the application of multivariate logistic regression analysis identified a significant connection between increased FBS, reduced HDL, and elevated LDL in patients and controls. A substantial association was also detected between cholesterol and HDL.

MiR-224 expression is controlled by NF-B inflammatory signaling pathway and TGF- signaling pathways [28]. miRNA expression profile studies in type 2 diabetes, according to (Zhu & Leung, 2015) [29], found 40 significantly strongly and consistently dysregulated miRNAs. Eight miRNAs might be blood biomarkers, whereas two miRNAs show considerable tissue-specific regulation and could be tissue biomarkers**.**

In the present study, the ROC curve for micro-RNA 224 level detected in the T2DM research sample exhibited that sensitivity = 69.0; specificity = 90.5; PPV 87.3%; NPV of 75.4%; Total accuracy of 80; area under the curve was 0.698. This is consistent with the ROC analysis of [30], which implies that circulating miRNAs could be used in medical settings as a potential technique for anticipating the onset of T2DM. A collection of miRNAs, including miR-15a, miR-29b, miR-126, miR-223, and miR-28-3p, were also disrupted before the onset of T2DM, according to a prior study [31]. More recent research found that ROC analysis detected 9 miRNAs that, when combined with HbA1c, have a sensitivity of 76.6%, a specificity of 80.9%, and an accuracy of 80.0% with a higher predictive value in the early diagnosis of type 2 diabetes (AUC = 0.8342) than HbA1c alone (AUC = 0.6950) [30].

Moreover, ROC analysis of pooled diabetic groups showed that miR-224 may discriminate between normal diabetic and control groups [27]. With this perspective, circulating miRNAs are considered a remarkable novel tool for predicting T2DM development in clinical practice.

MicroRNA-224 expression was also observed to have a highly significant correlation with WBC, neutrophil, HbA1C%, FBS, PPBS, INR, HDL, LDL, and eye complications. According to recent research, variable concentrations of circulating miRNAs may reproduce intriguing potential for T2DM therapy monitoring, prognosis, and diagnosis [32]. Increasing evidence assumes that multiple miRNAs were disturbed in T2DM cases and may have a role in the pathogenic pathways that lead to the development of diabetic complications [33]. The expression of several miRNAs relevant to the etiology of T2DM was explored and compared: those with newly diagnosed T2DM, those with pre-T2DM, and those with T2DM susceptibility but normal glucose tolerance. T2DM patients showed remarkably higher miRNA expression than T2DM susceptible people, according to the study [34].

Conclusion

Circulating miRNAs could be used in medical settings as a potential technique for anticipating the onset of T2DM. It may be used as a novel marker for the detection of diabetic complications.

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Conflict of interest: None

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Ethics statement: This paper was approved and had IRP approval by ethics committee of GOTHI (General Organization of Teaching Hospital and Institution). The code and number of approval was (NO: IDE00288).

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