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ORIGINAL ARTICLE

Expression profile of microRNAs may be promising in diagnosis of proliferative diabetic retinopathy: an Egyptian study

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Abstract

Purpose Diabetic retinopathy (DR) is still a leading cause of blindness. The role of miRNAs in diabetic retinopathy still needs more research. We aimed at validating the role of circulating miRNAs: 21,181C and 1179 in early detection, dynamic monitoring, and management of DR.

Methods Whole blood samples were collected from 180 diabetic patients and 60 normal individuals as control. The diabetic patients were subdivided into 60 subjects without retinopathy, 60 with non-proliferative diabetic retinopathy (NPDR), and 60 with proliferative diabetic retinopathy (PDR). Gene expression of miR-21, miR-181c, and miR-1179 were estimated in each sample using two-step reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR).

Results MicroRNA 181c and miRNA 1179 were significantly higher among PDR group compared to NPDR,W DM and control groups, while no significant difference was detected regarding miRNA 21. The areas under the receiver operating characteristic (ROC) curves of the validated two-serum miRNAs were 0.983 and 0.927 respectively. Combination of miRNA 1179 and miRNA 21 improved the accuracy rate to 90%. Combination of miR-181c and miR-1179 possessed high ability to discriminate between PDR and NPDR with an accuracy rate of 100%.

Conclusion MicroRNAs play a role in pathogenesis of diabetic retinopathy. In our study, miR-181c and miR-1179 were significantly high in PDR patients compared to NPDR and controls hence can be used to anticipate and follow up the progression. MicroRNA antagonists or mimics can be tried as new medications to modify DR by reducing the rate of progression, and subsequent blindness.

Keywords MicroRNAs · Proliferative diabetic retinopathy · miRNA181c · miRNA1179 · miRNA21

Introduction

Diabetic retinopathy (DR) is a common complication of DM and it may lead to complete blindness. It has long been known as a microvascular disease [1]. Clinically, DR has two stages: non-proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR). NPDR is the early stage and characterized by increased vascular permeability and the patient may be asymptomatic. PDR, more advanced stage, is characterized by neovascularization. The patient may complain from severe vision impairment because of vitreous hemorrhage or tractional retinal detachment [2]. Vascular endothelial growth factor (VEGF) and hypoxia-induced factor 1 (HIF-1α) are important factors for angiogenesis condition [3, 4].

MicroRNAs (miRNAs) are a class of highly conserved 19-25 nucleotide noncoding RNAs that regulate gene expression at the posttranscriptional level [5]. Recently, miRNAs have been demonstrated to play an important role in diabetes and its complications [6]. Serum miR-21, miR-181c, and miR-1179 levels could be sensitive and cost-effective biomarkers for the early detection of proliferative DR (PDR). Thus, investigation into the circulating levels of miRNAs in samples obtained from patients with different stage disease is guaranteed [7].

miR-181c targets many signaling pathways and cellular processes that are critical in diabetes-impaired angiogenic responses to ischemia. This includes HIF-1α stabilization and VEGFA expression in the early phase. In the late phase, it

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promotes (phosphatase and tensin homolog) PTEN inhibition and collagen dysregulation [8]. miR-21 was reported to target PTEN, leading to activation of HIF-1α and VEGF expression [9]. Also, miR 1179 has been listed as candidate miRNA being over expressed in cases of diabetic retinopathy [10]. Although recent studies have revealed a link between the expression of miRNAs and the development of DR, these studies mainly focused on miRNAs expressed in tissues of animal model. Few studies have specifically examined the role of miRNAs in DR patients [11]. It is likely that deepening our understanding in this field, and hopefully, more experiments to prove evidence for the correlation between miRNAs and DR are needed in the future [12].

In this study, we aimed to investigate the role of plasma (miR181c,miR1179 miR-21) in the pathogenic process and in diagnosing the severity of DR. Our study might provide a valuable reference for the clinical diagnosis of patients with DR.

Patients and methods

This prospective comparative study was conducted at Departments of Ophthalmology and Medical Biochemistry & Molecular Biology, Faculty of Medicine, Benha University, in the period between May 2015 and April 2019. This study protocol was approved by the Local Ethical Committee at Benha University. The study included 240 Egyptian adults (180 diabetic cases, 60 control) and the sample size was calculated using EPI-Info (Epidemiological information package) software version 7.2.4.

- 180 diabetic cases were further subdivided into three groups:
- **Group I**: DM group without retinopathy (WDM) included 60 patients diagnosed according to World Health Organization (WHO) guidelines [13] and the diagnosis validated at recruitment by patient's history and review of the medical records. The exclusion criteria were gestational diabetes and severe advanced diabetic complications defined as being registered blind.
- **Groups II and III**: diabetic retinopathy groups included 60 patients with non-proliferative diabetic retinopathy and 60 patients with proliferative diabetic retinopathy.

Retinopathy diagnosis was made by clinical examination done by a retina specialist, fluorescein angiography (FA), and macular optical coherence tomography (OCT) of both eyes. The worst eye defined the level of retinopathy [14]. The exclusion criteria were as follows: history of previous laser photocoagulation, presence of cataract, glaucoma, history of eye trauma or previous ocular surgery. None of these patients was on anti-vascular endothelial growth factor (anti-VEGF) or laser therapy.

- **Group IV**: for comparative purposes, the study also included 60 normal individuals of cross matched age attending the diabetic clinic to have blood glucose testing and proved to be non-diabetic. They gave blood samples as control group.

All study individuals signed written fully informed consent including the investigations to be performed.

Measurements

Measurements

The following data were recorded and coded: age (years), sex, height, weight, body mass index (kg/m^2), duration of DM, and type of DM (IDDM &NIDDM). Additionally, HbA1c (%) was measured at recruitment.

Blood sample collection

Peripheral venous blood samples were collected in vacutainer tubes containing EDTA under complete aseptic conditions then immediately frozen at −80°C till molecular assay of microRNA was done according to manufacturer's instructions.

Total RNA extraction

Total RNA was extracted from whole blood samples using RNeasy kit; Qiagen, Valencia, CA, according to manufacturer's instructions.

Molecular assay of microRNAs

Two-step RT-PCR for hsa-miR-21, hsa-miR-181c, and has-miR-1179 by real-time PCR was performed using Qiagen miScript preAMP RT-PCR kit (Qiagen GmbH Hilden, Germany) for conversion of microRNA to cDNA in a G-storm thermocycler (UK). Then, amplification and quantification of RNAs was done by real-time PCR in ABI7900 (Applied Biosystem, USA) using SuperReal Premix Plus Quanti Tect. Kit, SYBR Green (Tiagen, Shanghai) according to manufacturer's instructions and using the specific primers for each. Real-time cycler conditions were 95°C for 15 min for initial denaturation, followed by 40 cycles of 95°C within 30 s for

denaturation, 55°C for 1 min for annealing, and 72°C for 1 min for extension step. The target sequences were hsa-miR-21, hsa-miR-181c, and has-miR-1179, and the calibrator sample is normal lens. The identification of suitable housekeeping genes is a crucial step for deriving reproducible results when investigating the differential expression of miRNAs [15]. The reference gene (housekeeping gene) was RNU6B (U6) as it is the most commonly used gene to normalize miRNA RT-qPCR data [16].

Sequence name	Forward primer	Reverse primer
miR-21 were	5′- CTC AAC TGG TGT CGT GGA GTC GGC AAT TCA GTT GAG TCA ACA TC -3	5′- ACA CTC CAG CTG GGT AGC TTA TCA GAC TGA -3′
miR-181c	5′- ACA CTC CAG CTG GGA ACA TTC AAC CTG TCG -3′	5′- CTC AAC TGG TGT CGT GGA GTC GGC AA T TCA GTT GAG ACT CAC CG -3′
miR-1179	5′-ACA CTC CAG CTG GGA AGC ATT CTT TCA TT-3′	5′-AAC GCT TCA CGA ATT TGC GT -3′
RNU6B	5′- CTC GCT TCG GCA GCA CA -3′ [15]	5′-AAC GCT TCA CGA ATT TGC GT -3′ [17]

The threshold cycle (CT) served as a tool for the calculation of the starting template amount in each sample, and gene fold expression changes were calculated using the equation $2^{-\Delta\Delta ct}$ and because of the relative nature of quantification using the $2^{-\Delta\Delta ct}$ method, adjustment was required for each sample. Briefly, cDNA was diluted 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}-fold prior to amplification by real-time PCR and a standard curve was derived in order to obtain optimal amplification conditions [18].

Statistical analysis

The collected data were tabulated and analyzed using SPSS version 16 software (Spss Inc, Chicago, ILL Company). Categorical data were presented as number and percentages, using Fisher's exact test (FET) for their analysis. Continuous data were expressed as mean ± standard deviation, median, IQR, and range. Data were tested for normality using Shapiro-Wilks test, assuming normality at $p>0.05$. Differences between groups were tested using ANOVA (F test) for normally distributed variables or Kruskal-Wallis test for non-parametric ones. Significant ANOVA or Kruskal-Wallis was followed by post hoc multiple comparisons using Bonferroni test to detect the significant pairs. ROC curve analysis was used to detect cut-off values for the studied miRNAs with optimum sensitivity and specificity in prediction of PDR. The accepted level of significance in this work was stated at 0.05 (p <0.05 was considered significant).

Results

This study included 240 cases; 60 patients had PDR, 60 patients had NPDR, 60 diabetic patients without diabetic retinopathy, and 60 non-diabetic individuals as a control group. Details of the socio-demographic characters of enrolled patients and controls are shown in Tables 1, 2, and 3, which showed statistically significant increase in the duration of DM (13.5 ±5.4 years) in PDR group compared to diabetic patients without retinopathy (WDM group) (8.5±3.02 years). There were non-significant differences between them regarding age, weight, height, BMI, HbA1c, sex, and type of DM.

Comparing the groups regarding the studied markers showed that the mean values expressed in FRU of miRNA181C and miRNA1179 were significantly higher among PDR group (26654.4 ± 12857.8 and 22202.4 ±

Table 1 Comparison of the studied groups regarding socio-demographic characters

Variable	Controls (n=60)			DM (n=60)			NPDR (n=60)			PDR (n=60)			ANOVA (F test)	p
	Mean	±SD	Range	Mean	±SD	Range	Mean	±SD	Range	Mean	±SD	Range		
Age (ys)	47.1	10.3	29–65	50.9	9.6	34–65	48.9	8.2	41–65	44.9	8.2	33–57	**0.78**	**0.51 (NS)**
Weight (kg)	82.0	4.92	75–89	88.7	10.31	75–105	90.0	9.88	74–102	89.1	7.18	77–100	**1.93**	**0.14 (NS)**
Height (m)	1.66	0.06	1.57–1.75	1.66	0.10	1.54–1.83	1.67	0.08	1.55–1.8	1.67	0.07	1.55–1.8	**0.028**	**0.99 (NS)**
BMI (kg/m^2)	29.6	3.16	25.3–34.4	32.4	5.88	23.3–44.3	32.5	5.71	22.8–42.4	32.0	3.78	26.3–39.5	**0.78**	**0.51 (NS)**
Duration of DM (Ys)	----------			8.5	3.02	4–13	12.2	2.09	9–15	13.5†	5.40	7–25	**4.73**	**0.017 (S)**
HbA1c	----------			7.79	2.78	3.9–12.1	7.30	2.80	4.1–12.2	8.70	2.56	5.2–12.2	**0.68**	**0.51 (NS)**

†Significant in comparison with the DM

Table 3 Comparison of the studied groups regarding type of DM

			Groups			Total
			DM	NPDR	PDR	
Type of DM	IDDM	Count	24	36	18	78
		% within groups	40.0%	60.0%	30.0%	43.3%
	NIDDM	Count	36	24	42	102
		% within groups	60.0%	40.0%	70.0%	56.7%
Total		Count	60	60	60	180
		% within groups	100.0%	100.0%	100.0%	100.0%

FET=1.86; p=0.53 (NS)

10606.4 respectively) in comparison with NPDR (3756.7 ±1785.9 and 6736.4 ±3271.1 respectively), WDM (4346.0 ±2110.4 and 7523.5±3564.6 respectively), and control groups (4958.8± 2407.9 and 5454.9 ±2648.8 respectively) (p <0.001), while no statistically significant difference was detected regarding miRNA21 whose mean values (FRU) were 9354.9 ±4542.5 for PDR group, 4811.0 ±2336.2 for NPDR group, 5773.3 ±2803.2 for DM without retinopathy group, and 5345.6 ± 2595.6 for the control group (Table 4 and Figures 1, 2, and 3). Relative quantitation of the studied markers shows that miRNA 181c level was 5.39 folds in PDR, 0.76 folds in NPDR, and 0.8 folds in DM without retinopathy, respectively, compared to the control group.

While miRNA 1179 level was 3.97 folds in PDR, 1.3 folds, in NPDR, and 1.52 folds in DM without retinopathy, respectively, compared to the control group.

Finally, miRNA 21 level was 1.69 folds in PDR, 0.9 folds in NPDR, and 1.09 folds in DM without retinopathy respectively compared to the control group. Because samples of control group are used as calibrators, the expression levels are set to one. Gene expression levels were plotted as log10 values (log10 of 1 is 0), so the expression level of the calibrator samples appears as 0 in the graph [12] (Figures 4 and 5).

The receiver operating characteristic (ROC) curve analysis shows that miRNA181C ≥ miRNA1179 significantly predicts PDR. For miRNA 181C: (AUC=0.983, 95%CI =0.94–1.0), sensitivity was 90% and specificity was 100%. For miRNA1179: (AUC=0.927, 95%CI =0.82–1.0), sensitivity was 90% and specificity was 80%. In combining markers, the sensitivity improved to 100% for (miRNA 181+ miRNA 1179), 90% for both (miRNA 181+ miRNA 21) and (miRNA 1179+ miRNA 21). When combining all markers together, the accuracy reached 100% (Figure 6).

Discussion

DR is a highly specific vascular complication and a sight-threatening problem related to diabetes. PDR is characterized by gradually progressive alterations in the retinal microvasculature, leading to retinal hypo perfusion and increased vascular permeability [19]. Thus, detecting a novel factor to monitor the progression of the proliferation of vascular endothelial is very important. Few studies had listed three PDR-associated miRNAs, including miR-21, miR-181c, and miR-1179. miR-181c inhibition promoted tubule formation and VEGFA expression [12, 20].

Table 2 Comparison of the studied groups regarding sex

			Groups				Total
			Controls	DM	NPDR	PDR	
Sex	Male	Count	30	18	36	42	126
		% within groups	50.0%	30.0%	60.0%	70.0%	52.5%
	Female	Count	30	42	24	18	114
		% within groups	50.0%	70.0%	40.0%	30.0%	47.5%
Total		Count	60	60	60	60	240
		% within Groups	100.0%	100.0%	100.0%	100.0%	100.0%

FET=3.4; p=0.39 (NS)

Table 4 Comparison of the studied groups regarding the studied markers

Variable	Controls (n=60)			DM (n=60)			NPDR (n=60)			PDR (n=60)			Kruskal-Wallis test	p
	Mean	±SD	Range	Mean	±SD	Range	Mean	±SD	Range	Mean	±SD	Range		
miRNA181C (FRU)	4958.8	2407.9	1234–7895	4346.0	2110.4	1074–7027	3756.7	1785.9	975–6071	26654.4	12857.8*†‡	6713–42080	**21.8**	**<0.001 (HS)**
miRNA1179 (FRU)	5454.9	2648.8	1357–8685	7523.5	3564.6	1968–12258	6736.4	3271.1	1676–10725	22202.4	10606.4*†‡	5701–35505	**17.4**	**<0.001 (HS)**
miRNA21 (FRU)	5345.6	2595.6	1330–8511	5773.3	2803.2	1437–9192	4811.0	2336.2	1197–7660	9354.9	4542.5	2328–14894	**6.85**	**0.077 (NS)**

*Significant in comparison with the controls
†Significant in comparison with the DM
‡Significant in comparison with the NPDR

We have studied the three miRNAs including miR-21, miR-181c, and miR-1179 in predicting PDR out of NPDR which is very important for detecting the progression of patients with NPD developing to PDR.

Our results indicated the mean values expressed in FRU of miRNA181C and miRNA1179 were significantly higher among PDR group (26654.4 ± 12857.8 and 22202.4 ± 10606.4 respectively) in comparison with NPDR (3756.7±1785.9 and 6736.4 ± 3271.1 respectively), DM (4346.0±2110.4 and 7523.5±3564.6 respectively), and control groups (4958.8± 2407.9 and 5454.9 ±2648.8 respectively) (p <0.001) while no statistically significant difference was detected regarding miRNA21 whose mean values (FRU) were 9354.9 ±4542.5 for PDR group, 4811.0 ±2336.2 for NPDR group, 5773.3 ±2803.2 for DM group, and 5345.6 ±2595.6 for the control group (Table 4 and Figures 1, 2, and 3). In combining markers, the sensitivity improved to 100% for (miRNA 181c+ miRNA 1179), and 90% for both (miRNA181+ miRNA 21) and (miRNA 1179+ miRNA 21). When combining all markers together, the accuracy reached 100% (Figure 6).

Consistent with our results, Qing et al. [7] reported an accuracy rate of the three-miRNA (mir-181c and miR-1179 and miR-21) profile was 82.6%.

Previous studies had revealed the overexpression of mir RNA 181c in the pathogenesis of retinopathy [8 and 12, 21].

Unlike the results obtained by us, studies revealed a significant increase in miR-21 expression in the vitreous humor in proliferative diabetic retinopathy [22].

Demographic data revealed that there were not any significant differences between the three groups' (DMC, NPDR, and PDR) HbA1c levels. These observations agree with that reported by Ma et al. [23]. On the other hand, Raum et al. [24] reported that elevated levels of HbA1C were associated with increased risk of DR.

Conclusion

Since DR may continue to progress even in patients with controlled diabetes, there was a need to search for new biomarkers that can help identify high-risk DR patients and start therapy. MicroRNAs play a role in pathogenesis of diabetic retinopathy specially angiogenesis. In our study, miR-181c and miR-1179 were found to be significantly high in PDR patients compared to NPDR patients and controls so they could be used effectively to anticipate and follow this disease. MicroRNA antagonists or mimics can also be tried as new medications to modify the occurrence and progression of DR. This can reduce the rate of progression of DR, and the development of blindness.

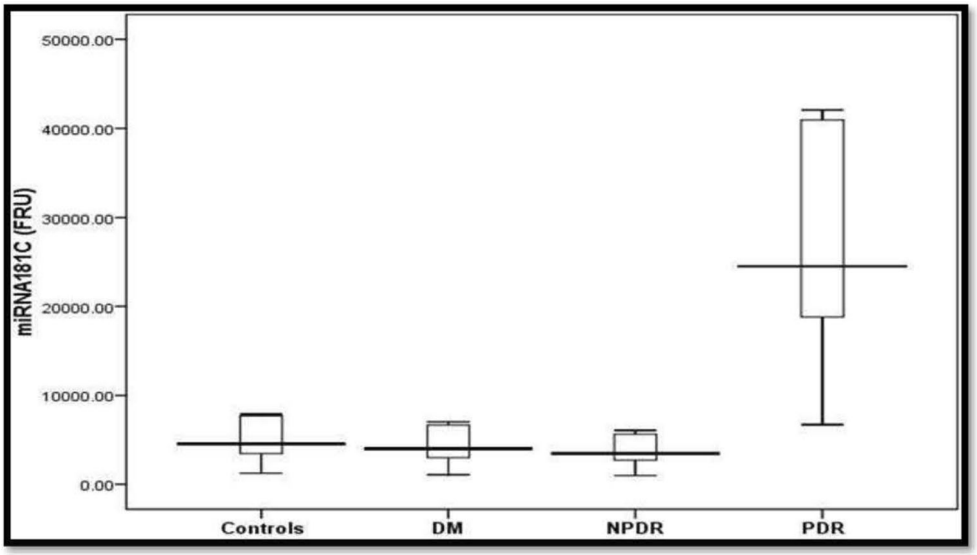

Fig. 1 Box plot showing the median and interquartile range (IQR) of miRNA181C among the studied groups. The median values were (4550.5, 4004.0, 3469.5, 24503.5) and the IOR values were (3178.3–7685.0, 2765.3–6686.0, 2478.5–5686.8, 17225.5–41172.3) among control, diabetes mellitus without retinopathy, non-proliferative diabetic retinopathy and proliferative diabetic retinopathy groups respectively

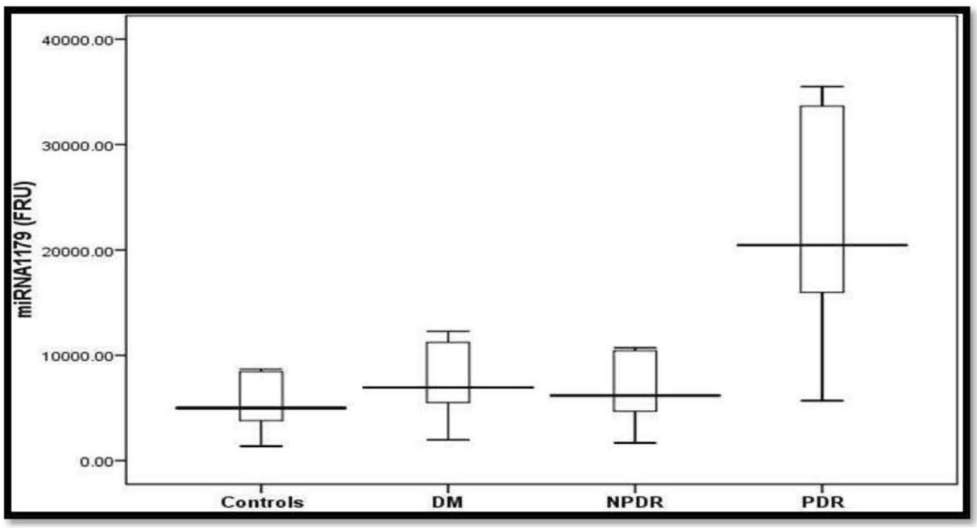

Fig. 2 Box plot showing the median and interquartile range (IQR) of miRNA1179 among the studied groups. The median values were (5005.5, 6957.0, 6181.5, 20470.5) and the IOR values were (3496.5–8454.0, 4991.8–11319.8, 4317.8–10440.0, 14541.8–33874.8) among control, diabetes mellitus without retinopathy, nonproliferative diabetic retinopathy and proliferative diabetic retinopathy groups respectively

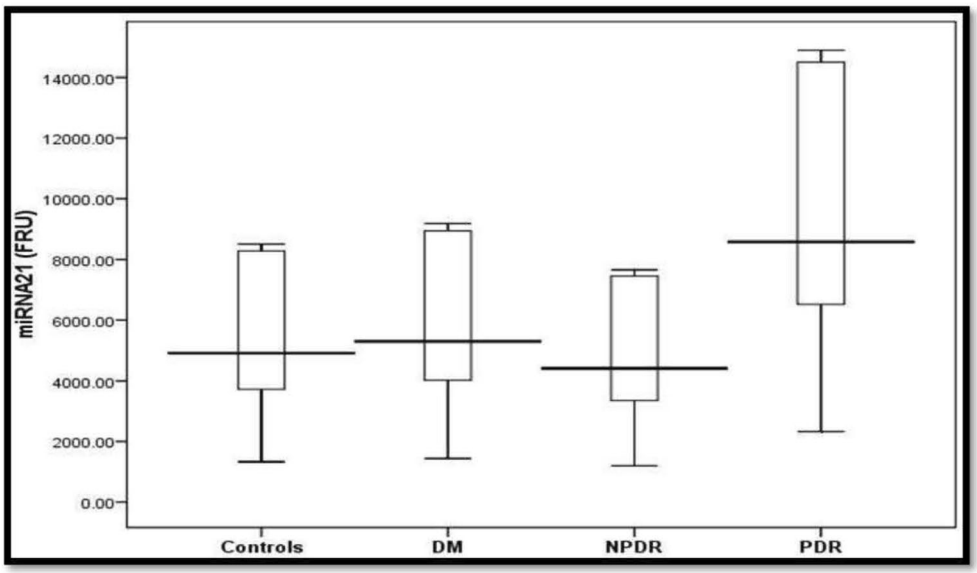

Fig. 3 Box plot showing the median and interquartile range (IQR) of miRNA21 among the studied groups. The median values were (4905.5, 5298.0, 4415.0, 8584.5) and the IOR values were (3426.5–8284.0, 3700.5–8947.0, 3083.5–7456.0, 5996.0–14498.0) among control, diabetes mellitus without retinopathy, non-proliferative diabetic retinopathy and proliferative diabetic retinopathy groups respectively

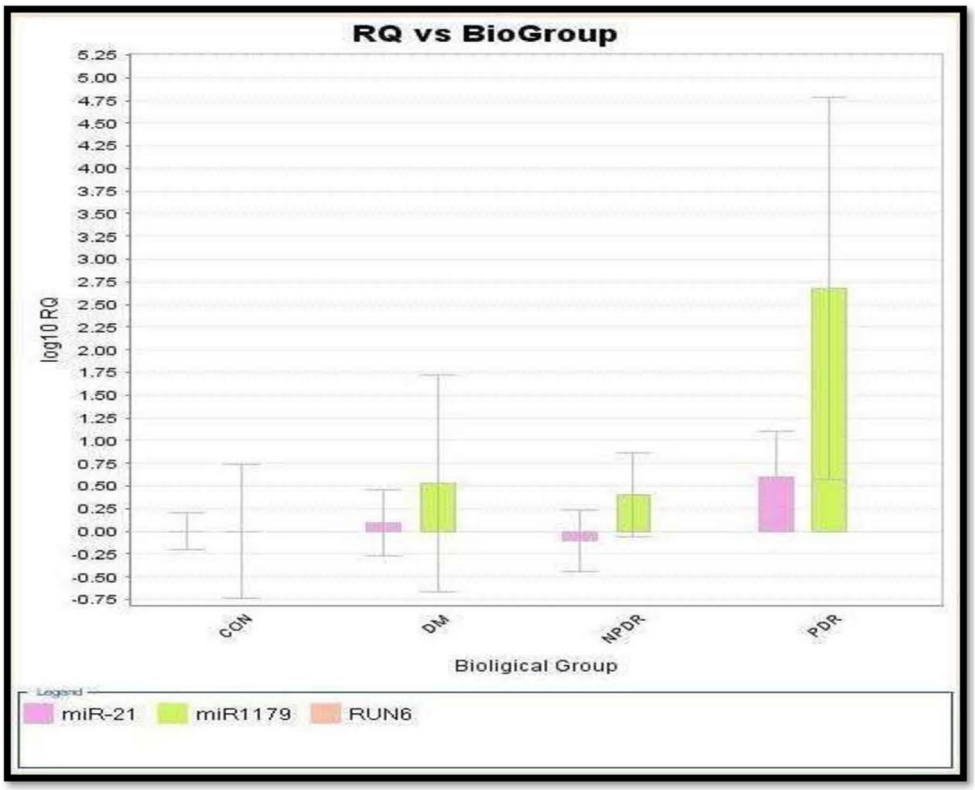

Fig. 4 Expression pattern of miR-21 and miR-1179 (RQ) in the studied groups compared to control group plotted as log 10 value. miRNA 1179 level was 3.97 folds in PDR, 1.3 folds in NPDR, and 1.52 folds in W DM, respectively, compared to the control group. While miRNA 21 level was 1.69 folds in PDR, 0.9 folds in NPDR, and 1.09 folds in W DM respectively compared to the control group. The control group is used as a calibrator so the expression levels of other groups are set to 1. As log 10 of 1 is 0, the expression level of the calibrator appears as 0 in the graph [12]

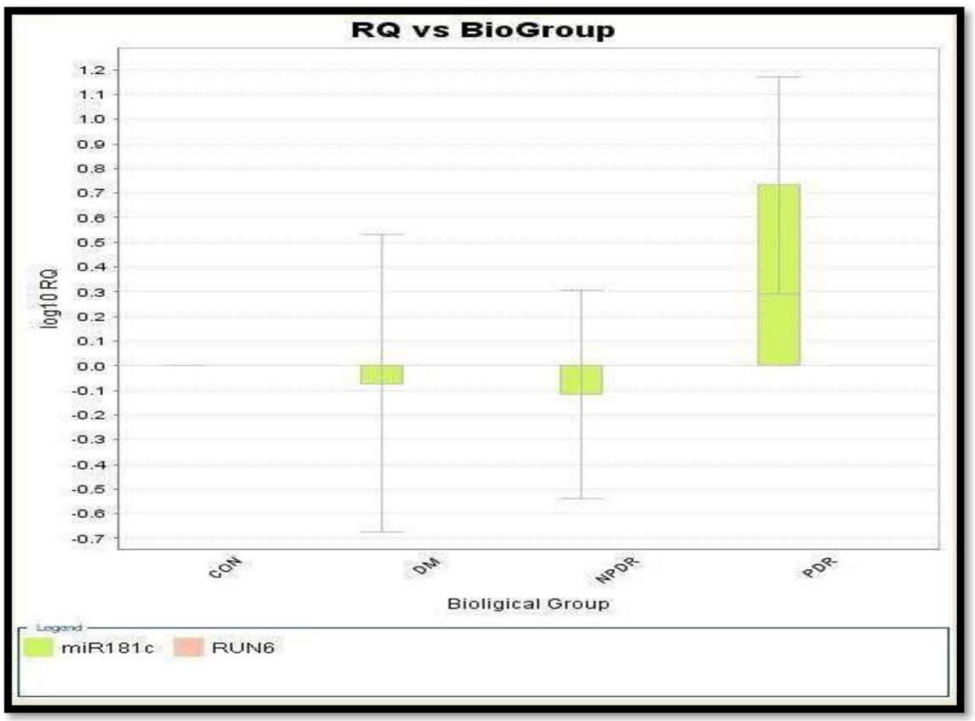

Fig. 5 Expression pattern of miR-181c (RQ) in the studied groups compared to control group plotted as log 10 values. Relative quantitation of the studied markers shows that miRNA 181c level was5.39 folds in PDR, 0.76 folds in NPDR, and 0.8 folds in WDM, respectively, compared to the control group

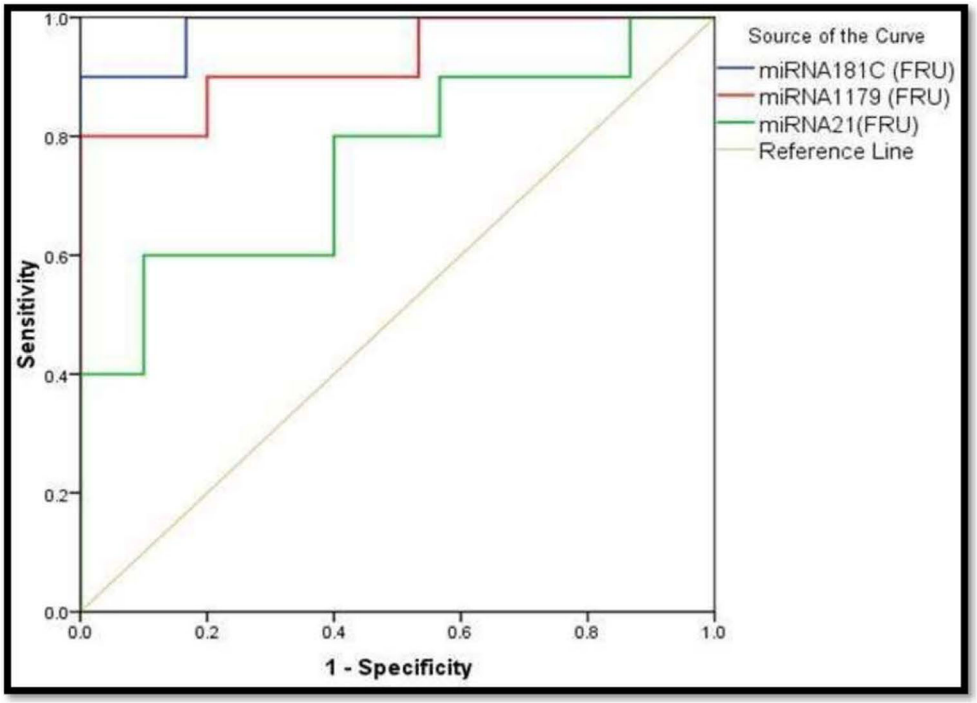

Fig. 6 ROC curve for the performance of the studied markers in the prediction of PDR. The receiver operating characteristic (ROC) curve analysis shows that miRNA181C ≥ miRNA1179 significantly predicts PDR. For miRNA 181C: (AUC=0.983, 95%CI =0.94–1.0), sensitivity was 90% and specificity was 100%. For miRNA1179: (AUC=0.927, 95%CI=0.82–1.0), sensitivity was 90% and specificity was 80%. In combining markers, the sensitivity improved to 100% for (miRNA 181c+ miRNA 1179), and 90% for both (miRNA181+ miRNA 21) and (miRNA 1179+ miRNA 21). When combining all markers together, the accuracy reached 100%

Availability of data and materials All relevant raw data will be freely available to any scientist wishing to use them for non-commercial purposes without breaching participant confidentiality.

Author contribution Tamer Ibrahiem Salem: collecting samples and clinical data, drafted the manuscript and revision of the manuscript

Nashwa Badr Eldin: collecting samples and clinical data, drafted the manuscript and revision of the manuscript

Naglaa Fathy Alhusseini: molecular biology technique

Omnia Alsaied Abdullah: conceived and designed the research, molecular biology technique, analyzed and interpreted the data, critical revision of the manuscript and corresponding author

Nashwa Elsayed Ahmed: rewrote the parts previously been commented on by the reviewers and needed to be corrected in the manuscript

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Declarations

Competing interests The authors declare no competing interest.

Animal research This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH publications 85-23, revised 2011). All protocols were approved by the institutional review board for animal experiments of the Faculty of Medicine, Benha University, Egypt.

Consent to participate and consent to publish The research protocol was accepted by the Medical Faculty Ethical Committee, Benha University. Before participation, written informed consents were received from all patients at the study initiation, both consent to participate in the study and consent to have the data published.

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