Original Article

MicroRNAs (−146a, −21 and −34a) are diagnostic and prognostic biomarkers for diabetic retinopathy

Hend Gouda Helal [a],[*], Mohammed H. Rashed [c], Omnia Alsaied Abdullah [b], Tamer Ibrahim Salem [a], Ahmed Daifalla [a]

[a] Department of Ophthalmology, Faculty of Medicine, Benha University, Benha, Egypt
[b] Department of Medical Biochemistry and Molecular Biology, Faculty of Medicine, Benha University, Benha, Egypt
[c] Department of Pharmacology and Toxicology, Faculty of Pharmacy, Al-Azhar University, Cairo, Egypt

ARTICLE INFO

ABSTRACT

Background: Diabetic retinopathy (DR) is implicated in blindness of diabetic patients. Early diagnosis of DR is very essential to ensure good prognosis. The role of microRNAs (miRs) as biomarker diagnostic tools in DR is not fully investigated. The present study aimed to find the relation between serum relative expression of microRNAs (miR-146a, miR-21 and miR-34a) and severity of DR and to what extent their expression pattern can be used as either diagnostic or prognostic.

Methods: Eighty type 2 diabetic patients were classified according to severity of DR into normal, mild, moderate, severe non-proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR). Serum relative expressions of miRNAs were evaluated by qPCR and statistically analysed in each stage using Analysis of Variance (ANOVA) followed by Tuckey-Kramer post-test.

Results: Serum relative expressions of miR-146a and miR-21 were increased with increased severity of DR. miR-34a decreased with the severity of DR. The expression pattern in each group in relation to normal fundus group could be diagnostic and prognostic where miR-146a was only increased in mild group and continued with the severity. In moderate group miR-21 start to increase along with slight decrease in miR-34a. In severe NPDR group along with highly increased levels of both miR-146a and miR-21, a marked decrease in miR-34a. In PDR group miR-34a was almost diminished along with very high levels of both miR-146a and miR-21.

Conclusions: miRs (−146a,-21 and-34a) are promising biomarkers in DR and can help to avoid disease progression.

Diabetes mellitus (DM) affects >350 million people worldwide and makes a considerable contribution to morbidity and mortality globally [1]. DM is a major emerging clinical and public health problem in Egypt with a prevalence of 5—10% in the 1990s [2]. It has been estimated that by the year 2025, nearly over 13% of the population over 20 years of age will have DM

At a glance of commentary

Scientific background on the subject

Diagnosis of diabetic retinopathy requires specialized resources and specially trained staff. In addition in most cases diagnosis is delayed as it is asymptomatic disease. Modulation of expression pattern of serum miRNAs (−146a, −21 and −34a) may be used as biomarkers for early diagnosis and staging of severity.

What this study adds to the field

The present study mainly include the possibility of helping diagnosis by non-invasive method depending on evaluation of circulating miRNAs. This can also lead to early diagnosis with subsequent avoidance of disease progression. Also, expression pattern of circulating miRNAs can be used as a prognostic tool.

[3,4]. DR is the leading cause of visual impairment or blindness in diabetic patients. Approximately 80% of all patients with DM duration of at least 10 years suffer from some degree of DR [5], whereas 20% of individuals with a history of diabetes do not develop DR regardless of glycemic exposure, indicating that genetic factors may play a role in the development of DR [6]. DR is classified into NPDR and PDR. By 2030, NPDR and PDR will be estimated to rise to 191.0 million [7]. Therefore, early detection of DR can reduce the high morbidity of this disease. Currently, formal diagnosis of DR and DR severity requires visualization of the retina which in turn requires specialized resources and specially trained staff [8]. To develop new therapeutic strategies for early stages of DR, new diagnostic tools are urgently needed. There are no means of detecting patients at-risk of diabetic complications. In this regard, circulating biomarkers could be useful [9]. Abnormal expression of miRNAs has been reported in DM and in murine models of DR [10—12]. However, the roles of miRNAs in DR have not been fully studied. So, the identification of potential-specific miRNAs biomarkers may help to predict or detect the development and the progression of DR at an early stage, and allow well timed intervention. miRNAs have been shown to play a key role in mammalian post-transcriptional gene expression [13,14]. These molecules have been found to be stable in many biological fluids, including human serum, plasma, urine, saliva, tears, aqueous humor, and vitreous humor [15]. The ability of miRNAs to alter or fine tune the expression of key regulators in various physiological processes and pathophysiological disease states makes them novel targets for diseases such as diabetic complications [16—18]. Several miRNAs have emerged as important regulators of particular aspects of DR pathology [19]. Based on comprehensive reviewing of the scientific literature concerning the use of miRNAs as a biomarkers and particularly in DR, the studied miRNAs (146a, 21, and 34a) were selected according to their biological availability in the different specimens (tissue, stool, plasma, and serum), but with inconsistent expression behavior among DM and DR specimens or with insufficient studies. miR-146a is upregulated in response to oxidative stress and inflammation in diabetic patients and play a protective role in progression of DR [17]. Also, serum samples from subjects with DR showed significantly increased level of miR-21 in patients with PDR [20]. On the other hand, altered expression of miR-34a is evident in several human pathologies, including cancer [21,22] and cardiovascular disease [23]. Its role in progression of DR is not studied till now. Few studies indicated the involvement of miR-34a in the proliferation and migration of retinal pigment epithelial (RPE) cells, important in vitreoretinopathy [24] but did not clarify its pattern of expression in different DR stages. There are a few published data regarding the frequency and patterns of aberrant miRNAs expression and their contribution to the development and progression of DR in humans. Therefore, the aim of this study was to use the expression pattern of miRs (−146a, −21, and −34a) as biomarkers for early detection of DR in addition to the underlying mechanisms. Also, to evaluate the expression of them in each stage of DR to help in and certify diagnosis by simple noninvasive methods. To our knowledge, this is the first direct, in-depth investigation on the patterns of expressions of serum miRs (−146a, −21 and −34a) in different stages of DR patients. Also, for the first time, the relation between the expressions of these miRNAs and the progression of DR is investigated.

Patients and methods

Subjects and experimental design

This study was conducted at Ophthalmology and Medical Biochemistry and Molecular Biology Department of Benha Faculty of Medicine and approved by Faculty ethics committee No. (ms 8-6-2019). It included 80 subjects' suffering from type 2 diabetes mellitus. They were diagnosed according to American Diabetes Association diagnostic criteria [25,26]. Additionally, age, sex, BMI (body mass index), and family history of diabetes were recorded. All of the subjects underwent a general physical examination. Informed consent from each subject after full explanation of the study was taken before being enrolled.

Diabetic patients (n = 80) with duration of diabetes ranged from 2 to 12 years were divided into 5 subgroups: Those with newly diagnosed type 2 diabetes mellitus without evident complication (n = 15, 5 males and 10 females); those with non-proliferative DR (n = 45) were classified equally into mild NPDR (7 males and 8 females) in which FA shows micro aneurysms and hemorrhages which appeared as hypofluorescent dots without or with minimal extent of macular edema by OCT. Moderate NPDR patients (6 males and 9 females) show blocked fluorescence by dot and blot hemorrhages, leaking and non-leaking micro aneurysms and co-existing macular edema by EA and OCT respectively. However, in patients with severe NPDR (8 males and 7 females), FA shows extensive blot hemorrhages with macular edema. The rest 20 patients (9 males and 11 females) were type 2 diabetic diagnosed as suffering from PDR by FA and OCT.

Patients with a history of liver cirrhosis, malignancy, infection, inflammation, bronchial asthma, heart disease, renal disease or any other eye diseases associated with retinal degenerations or oxidative stress such as high myopia

and age-related macular degeneration (ARMD) were excluded from the study.

From each patient in each group 6 ml blood was taken and centrifuged to separate the sera that kept at-80 °C till used for biochemical and molecular analysis including lab investigations of blood glucose, lipid profile, serum creatinine. Also, miRNAs were isolated, quantified and tested for purity before evaluation of relative expressions of miRs (146a, 34a and 21) using qPCR.

Biochemical analysis

Peripheral blood samples were collected following a 12-hour fasting. Glycosylated hemoglobin (HbA1c), blood glucose, total cholesterol, and triglycerides levels were estimated by routine enzymatic methods (Spinreact). TC, LDL, HDL and TG were measured using standard enzymatic methods. HDLc and LDLc were determined after precipitation of the apo B-containing lipoproteins using Friedewald formula [27]. Glycosylated hemoglobin (HbAlc) was estimated calorimetrically by using (Biosystems, Barcelona, Spain). Serum insulin and lipoprotein a (LPa) were measured by ELISA using the Quantikine human insulin and LPa assay kits (R and D Systems, Minneapolis, MN).

Serum miRNA assay

Total RNA was isolated from human serum by the standard TRIzol® Reagent extraction method (cat#15596-026, Invitrogen, Germany). Briefly, human serum samples were centrifuged at 2000 ×g for 5 min at 4 °C and then the pellets were homogenized in 1 ml of TRIzol® Reagent. Afterwards, the homogenized sample was incubated for 15 min at room temperature. A volume of 0.2 ml of chloroform per 1 ml of TRIzol® Reagent was added. Then, the samples were vortexed vigorously for 15 s and incubated at room temperature for 3 min. The samples were centrifuged for no more than 12,000 ×g for 15 min at 4 °C. Following centrifugation, the mixture was separated into lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA was remained exclusively in the aqueous phase. Therefore, the upper aqueous phase was carefully transferred without disturbing the interphase into a fresh tube. The RNA was precipitated from the aqueous phase by mixing with isopropyl alcohol. A volume of 0.5 ml of isopropyl alcohol was added per 1 ml of TRIzol® Reagent used for the initial homogenization. Afterwards, the samples were incubated at −20 °C for 30 min and centrifuged at not more than 12,000 ×g for 10 min at 4 °C. The RNA was precipitated which was often invisible before centrifugation, formed a gel-like pellet on the side and bottom of the tube. The supernatant was removed completely. The RNA pellet was washed once with 1 ml of 75% ethanol. The samples were mixed by vortexing and centrifuged at no more than 7500 ×g for 5 min at 4 °C. The supernatant was removed and RNA pellet was air-dried for 10 min. RNA was dissolved in diethylpyrocarbonate (DEPC)-treated water by passing solution a few times through a pipette tip.

Total RNA was treated with 1 U of RQ1 RNAse-free DNAse (Invitrogen, Germany) to digest DNA residues, re-suspended in DEPC-treated water. Purity of total RNA was assessed by the 260/280 nm ratio (between 1.8 and 2.1). Additionally, integrity was assured with ethidium bromide-stain analysis of 28S and 18S bands by formaldehyde-containing agarose gel electrophoresis. Aliquots were used immediately for reverse transcription (RT), otherwise stored at −80 °C.

Real time-PCR quantification of serum miRNAs

Quantification of miR-146, miR-21 and miR-34A were carried out StepOne™ Real-Time PCR System from Applied Biosystems (Thermo Fisher Scientific, Waltham, MA USA) was used to determine the human serum's cDNA copy number. Amplifications were performed using the miScript SYBR Green PCR kit (Qiagen GmbH) with a reaction mixture that included 12.5 μl 2X QuantiTect SYBR Green PCR Master Mix, 2.5 μl 10X miScript reference primer, 2.5 μl specific primers for miR-146a (355501996. Willowfort Co., Birmingham, UK), miR-34a (355502000. Willowfort Co., Birmingham, UK) and miR-21 (355501997. Willowfort Co., Birmingham, UK.), 5 μl RNase free water and 2.5 μl cDNA. The PCR conditions were used as follows: Initial denaturation at 95 °C for 15 min; then 94 °C for 15 s, 55 °C for 30 s and 70 °C for 30 s for 40 cycles. The melting curve was obtained from 65 °C to 95 °C. A quantitation cycle (Cq) value < 30 were obtained as good amplification but Cq value > 35 was regarded as impossible for amplification. qPCR primers are shown in Supplementary Table 1. Each sample was analyzed in duplicate. The relative expression of the miRNA, observed in patients in relation to control group, was calculated using the standard method ($2^{-\Delta\Delta CT}$) [28].

Statistical analysis

Data were analyzed with Graph Pad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). Quantitative data are expressed as the mean ± standard deviation, while threshold cycle (Ct) values were determined using the melting curve analysis to measure the expression of target miRNAs. Triplicate Ct values were averaged, and the relative expression level of each miRNA was calculated using the comparative threshold cycle (Ct) method (2-DDCt). All of the miRNA values are expressed as the mean ± SD. One-way analysis of variance was used to evaluate differences in serum miRNA levels between groups with Tukey-kramer post-test. While, One-way analysis of variance followed by Dunnett post-test was used to evaluate differences between % of miRNAs expressions in each group and that in normal group (diabetic with normal fundus). The relation between plasma miRNAs expression and metabolic parameters in the studied subjects has been tested. Differences were considered statistically significant at p value < 0.05.

Results

The demographic and biological characteristics of the study subjects

The clinical characteristics of the study subjects are presented in Supplementary Table 2. A total of 80 cases (matched by age, BMI and duration of diabetes) were included in the validation stage. There were no significant differences in family history of diabetes or in levels of TC, HDL, LDL, TG or HbA1c between the five groups.

Results of eye examination

Fluorescein angiography (FA) and Optical coherence tomography (OCT) images of the examined eyes were imaged and evaluated independently by 2 masked readers. Figure (1A) explains the fluorescein angiography of a diabetic patient with normal fundus with no angiographic abnormalities (no micro aneurysms, no neovessels or macular edema. OCT of these patients shows normal foveal contour with preserved foveal pit and no evidence of macular edema or macular traction [Fig. 1B].

Fluorescein angiography of mild NPDR fundus in diabetic patients shows micro aneurysms which in the form of hyperfluorescent dots around the blood vessels and some of them are leaking along with evidence of dot hemorrhages which appeared as hypofluorescent dots [Fig. 2A]. OCT for these patients revealed that 12 patients were not associated with macular edema (80%) and the rest 3 patients were associated with macular edema if there was leaking aneurisms near macular region (20%) [Fig. 2B].

Figure (3A) explains that fluorescein angiography of diabetic patients with moderate NPDR shows blocked fluorescence by dot and blot hemorrhages, leaking and non-leaking micro aneurysms and co-existing macular edema. There was no evidence of any neovascularization or IRMAs. OCT shows that 9 patients were associated with macular edema which was either center-involving or non-center involving (60%) [Fig. 3B], the remaining 6 patients were not associated with macular edema (40%).

Fluorescein angiography of diabetic patient with severe NPDR shows extensive blot hemorrhages in all retinal quadrants and vascular abnormalities in the form of tortuosity and congestion of veins or arterio-venous (A-V) crossing changes or venous beading and arteriolar narrowing with evidence of multiple micro aneurisms and no evidence of neovascularization [Fig. 4A]. OCT of 12 patients with severe NPDR
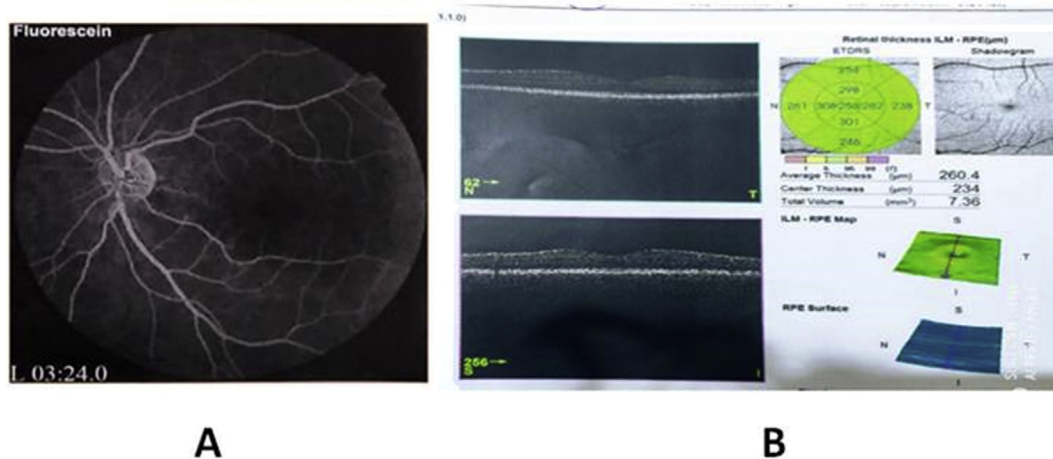

Fig. 1 Retinal fluorescein angiography (FA) and optical coherence tomography (OCT) images for the eye of diabetic patient with normal fundus. FA demonstrated normal fundus with no angiographic abnormalities (A). OCT of these patients shows normal foveal contour with preserved foveal pit and no evidence of macular edema or macular traction (B).
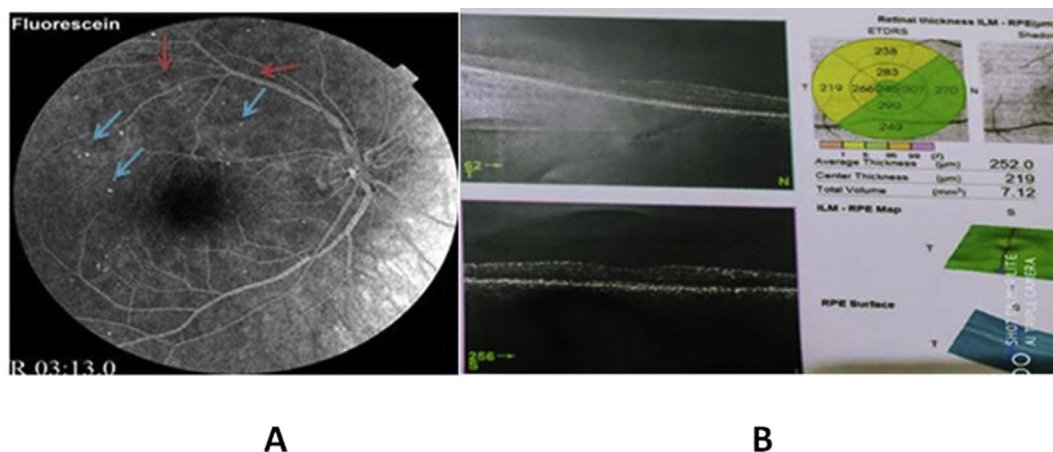

Fig. 2 Retinal fluorescein angiography (FA) and optical coherence tomography (OCT) images for the eye of diabetic patient with mild NPDR. FA showed micro aneurysms which in the form of hyperfluorescent dots around the blood vessels and some of them are leaking along with evidence of dot hemorrhages which appeared as hypofluorescent dots (A). OCT showed normal foveal contour with preserved foveal pit and no evidence of macular edema or macular traction (B).
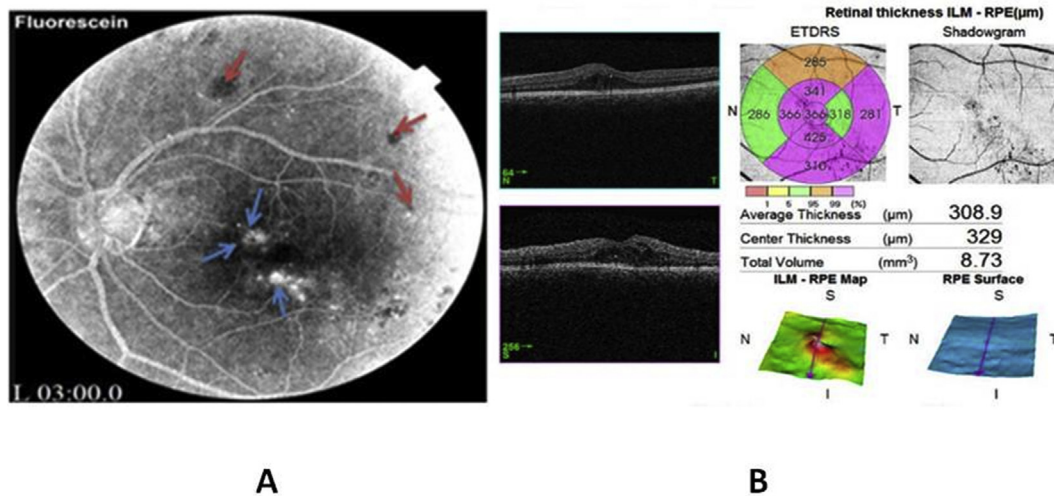
Fig. 3 Retinal fluorescein angiography (FA) and optical coherence tomography (OCT) images for the eye of diabetic patient with moderate NPDR. FA showed blocked fluorescence by dot and blot hemorrhages, leaking and non-leaking micro aneurysms and co-existing macular edema. (A). OCT showed macular edema which was either center-involving or non-center involving (B).
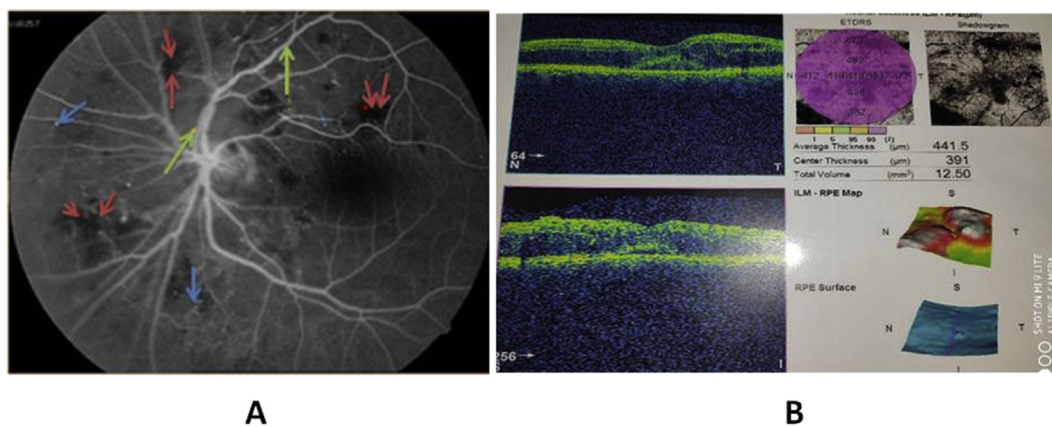

Fig. 4 Retinal fluorescein angiography (FA) and optical coherence tomography (OCT) images for the eye of diabetic patient with severe NPDR. FA showed extensive hemorrhages in almost all retinal quadrants (red arrows) with vascular abnormalities in the form of A-V crossing and tortuous veins (green arrows) and there are multiple microaneurysms (blue arrows) (A). OCT showed macular edema with neurosensory detachment (B).

show showed macular edema (80%) [Fig. 4B]. While the remaining 3 patients had no macular edema (20%).

Injection of fluorescein dye to PDR diabetic patients resulted in extensive leakage around disc from NVDs and/or NVEs with or without preretinal hemorrhages and sometimes there were areas of capillary drop out denoting ischemic areas [Fig. 5A]. Sometimes, there was widening of FAZ in early frames denoting ischemic maculopathy. OCT shows that 18 PDR patients has macular edema (90%) [Fig. 5B], Only 2 PDR patients showed no macular edema (10%).

Serum relative expression of miRs (−146a, −34a and −21) and severity of diabetic retinopathy

Data in Supplementary table (3) explain the level of expression of miRs (−146a,-34a, and −21) are 2.84 ± 0.17, 8.42 ± 0.20 and 1.86 ± 0.11 ($p < 0.05$) respectively in serum of diabetic patients with normal fundus. The level of miRNA146a expression is significantly increased in the order of mild < moderate < severe NPDR < PDR diabetic patients as it was found 3.77 ± 0.19, 6.99 ± 0.12, 10.90 ± 0.24 and 13.96 ± 0.15 respectively [Fig. 6A]. The relative expression of miR-34a in the serum of diabetic patients show an opposite pattern where the maximum value was in patients with normal and mild DR without any significant difference (8.42 ± 0.20 and 7.96 ± 0.17 respectively). As shown in Supplementary table (3) and Fig. (6B) this level is significantly declined upon disease progression to a significant level in each stage to be 5.99 ± 0.16 (moderate), 2.52 ± 0.16 (severe NPDR) and 0.84 ± 0.14 (PDR). Regarding the serum relative expression of miR-21 in different stages of DR, the expression is the same in patients with either normal or mild DR (1.86 ± 0.11 and 2.1 ± 0.12
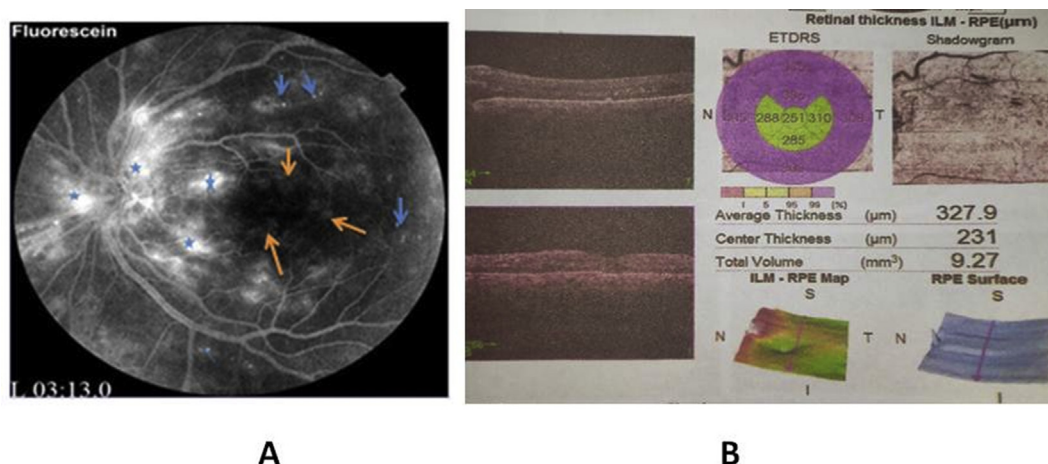
Fig. 5 Retinal fluorescein angiography (FA) and optical coherence tomography (OCT) images for the eye of diabetic patient with PDR. FA showed extensive leakage from NVDs and NVEs (blue stars), multiple leaking microaneurysms (blue arrows) and leakage around fovea denoting co-existing maculopathy (orange arrows) (A). OCT image showed non Centre —involving macular edema (B).

respectively [Supplementary Table 3 and Fig. 6C]. This level is directly increased in relation to severity to reach 4.78 ± 0.17, 8.28 ± 0.29 and 11.10 ± 0.20 in moderate, severe NPDR and PDR groups respectively.

Relation between serum relative expressions of miRs (−146a, −34a and-21) as biomarkers for staging and diagnosis of patients with diabetic retinopathy

The present study explains that extent of serum relative expression of miRs (−146a, −34a and-21) in relation to that of normal fundus patients ca be used as diagnostic and prognostic tool (Supplementary Table 4). Herein patients with mild DR show significantly increased extent of miR-146a to reach 132.7% compared to that in normal fundus with no significant changes in either miR-34a or miR-21 [Fig. 7A].

In moderate cases the serum relative expressions of both miR-146a and miR-21 is increased to reach 246% and 257% respectively compared to that in normal fundus patients along with marked decrease in the level of serum expression of miR-34a to be 71% of that in normal fundus group [Fig. 7B].

The data obtained in the present work revealed that severe NPDR patients showed higher extent of both miR-146a and −21 by 283% and 345% accompanied with highly significant decrease in the level of miR-34a (by 70%) that its level reaches 30%compared to patients with normal fundus [Fig. 7C].

Interestingly, data in the present work [Fig. 7D] explain that patients with PDR show aggressive decrease in serum relative expression of miR-34a to only 9% of that in patients with normal fundus. However, the levels of miR-146a as well as miR-21 are extensively increased to be 492% and 597% compared to patients with normal fundus [Fig. 7D].

In addition, the difference of the ratio of miR146-a to miR 34a and the ratio of miR-21 to miR- 34a were calculated to discriminate between the different degrees of DR. It was found that the difference for the ratio of miR 146-a: miR-34a was

36.1(for PDR: mild), 14.65 (for PDR: moderate) and 3.97 (for PDR: severe). Regarding miR-21: miR-34a, the difference for the ratio was 51.7, 17 and 4.15 for PDR: mild, PDR: moderate and PDR: severe, respectively.

Discussion

Diabetic retinopathy is a chronic and serious eye complication associated with DM, and people with DR present an increased risk of other microvascular and macrovascular complications associated with diabetes [29]. Retinal-cell dysfunction is characterized by a decrease in cell viability and increase in apoptosis [30]. Inflammation has a central role in pathogenesis of diabetic retinopathy [31].

In DR, early detection and treatment is of vital importance as it may prevent vision loss and blindness and screening for DR is cost-effective when compared with disability loss for people going blind in the absence of a screening program [32]. Thus, novel biomarkers to monitor the progression of the proliferation of vascular endothelium is very important for both the prevention and the updating of the therapy. It has been reported that the serum miRNA expression profiles for various diseases may serve as a potential biomarker for disease detection [33]. The present study was designed to investigate the patterns of expressions of serum miRs (−146a, −21 and −34a) in different stages of DR patients. Also, for the first time, the relation between the expression of three of them and the progression of DR is being investigated. In the present work fundi of diabetic patients were examined by FA and OCT and patients were categorized into normal fundus, mild, moderate, severe NPDR and PDR.

Data in present work revealed that there is direct relation between relative expression of miR-146a in the serum and severity of DR in the order of PDR > severe NPDR > moderate > mild > normal fundus patients. This finding
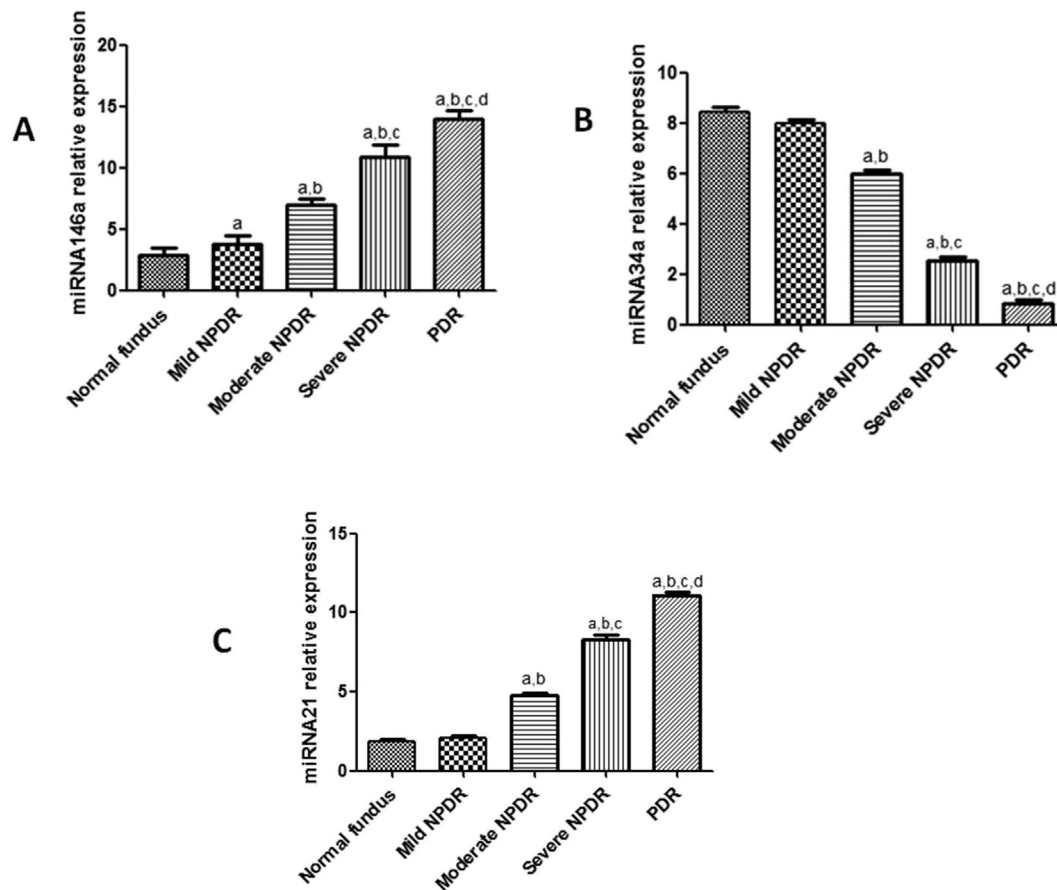
Fig. 6 Relative expression of miRNA-146a, miRNA-34a and miRNA-21 in the serum of normal fundus, NPDR (mild, moderate or severe) and PDR diabetic patients. The level of miRNA146a expression is significantly increased in the order of mild < moderate < severe NPDR < PDR diabetic patients (A). The relative expression of miRNA −34a in the serum of diabetic patients showed an opposite pattern where the maximum value was in patients with normal and mild DR without any significant difference, this level is significantly declined upon disease progression to a significant level in each stage (B). Regarding the serum relative expression of miRNA21 in different stages of DR, the expression is the same in patients with either normal or mild DR, this level is directly increased in relation to severity (C). Data are presented as mean ± SD and statistically analyzed using Graph Pad Software program by One Way ANOVA followed by Tukey Kramer post hoc test at $p < 0.05$. a: Significant difference from normal fundus group. b: Significant difference from mild group. c: Significant difference from moderate group. d: Significant difference from severe NPDR group.

is strongly supported by the study of Feng et al. [34]. Also, it has been reported that serum miR-146a was elevated in T2DM subjects compared to healthy controls [35]. The relation between the expression of miR-146a and activation of NF-Kb (inflammatory mediator) with progression of DR can explain its upregulation [36]. In addition, up-regulation of several NF-kB responsive miRNAs, including miR-146 in the retina and retinal endothelial cells (RECs) from Streptozotocin (STZ)-induced T1DM rats [37]. Furthermore, it has been suggested that miR-146a down-regulation could be a key mechanism for increased extracellular matrix protein production in diabetes [38].

In the present study miR-21 expression in the serum of diabetic patients was increased along with increased severity of DR. The highest level was in PDR group and the lowest one was in patients with normal fundus. Previous studies have shown increased level of miR-21 with chronic inflammation and subsequent endothelium impairment, pericyte loss,

increased capillary degeneration, and vascular leakage in DR [39,40]. miR-21 was increased in the vitreous humor of patients with proliferative DR, supporting that miR-21 may play a role in DR [41]. On the other hand, miR-21 is overexpressed in both DR and diabetic nephropathy (DN) and contributes to the pathogenesis of DR by enhancing inflammation ([17,42,43]. Moreover, it was found that increased levels of miR-21 in the vitreous are associated with retinal fibrosis, including PDR and proliferative vitreoretinopathy (PVR) [41]. Herein, it could be explained that miR-21 is considered as a prognostic biomarker for progression of diabetic retinopathy.

Although the role of miR-34a has been studied in some disease situations particularly carcinogenesis, very few studies tested its role in eye diseases. Also, this is the first study aimed to test its role in progression and severity of DR. The obtained data from the present work revealed that, diabetic patients with normal fundus show the highest level
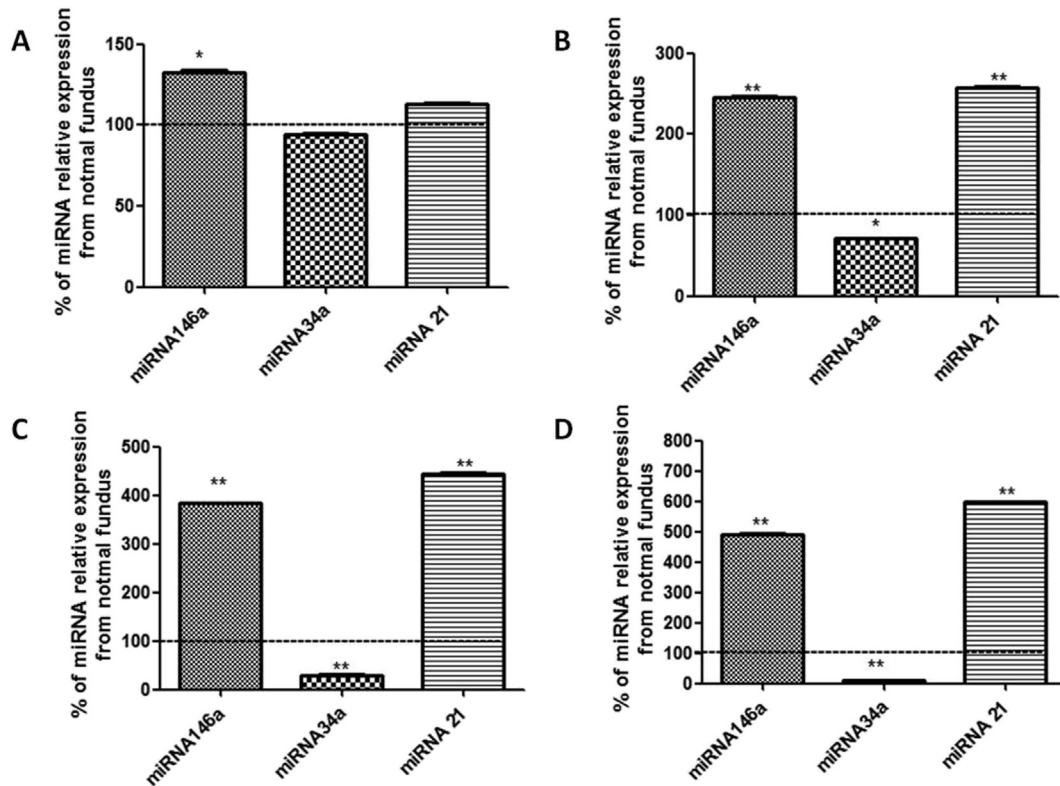
Fig. 7 % Relative expression of miRNAs (−146a, −34a and −21) in diabetic patients showing mild, moderate, sever NPDR and PDR compared to those with normal fundus. Patients with mild DR showed significant increase extent of miRNA −146a compared to that in normal fundus with no significant changes in either miRNA −34a or miRNA −21 (A). In moderate cases the serum relative expressions of both miRNA-146a and miRNA-21 is significantly increased compared to that in normal fundus patients along with marked decrease in the level of serum expression of miRNA −34a (B). In severe NPDR patients the extents of both miRNAs (−146a and −21) showed highly significant level of increase accompanied with highly significant decrease in the level of miRNA −34a compared to patients with normal fundus (C). Patients with PDR showed aggressive decrease in serum relative expression of miRNA-34a than that in patients with normal fundus, however the levels of miRNA −146a as well as miRNA −21 are extensively increased compared to patients with normal fundus (D). *: Significant difference from normal fundus group at $p < 0.05$. **: Highly significant difference from normal fundus group at $p < 0.01$.

of serum miR-34a expression. These data can be supported by previous studies indicated increased expression of miR-34a in experimental and human diabetes, which in turn is linked to hyperglycemia-induced vascular dysfunction [44,45], Oxidative stress [46,47], and apoptosis [21]. Thounaojam et al. (2019) explained that increased oxidative/nitrative stress plays a causal role in retinal vascular dysfunction and hyperglycemia-induced premature senescence [48].

This expression was declined with the progression of the disease and reaches its minimal level in diabetic patients with PDR. The decreased expression of miR-34a in the present work may be explained depending on the previous findings of the relation between increase oxidative stress and NF-kB activation and its effect on p53 activation (inducer of miR-34a expression). In this regard, p53 activation led to induction of the miR-34a, which sensitized beta cells to apoptosis and inhibited the insulin exocytosis pathway, resulting in impaired insulin secretion [49]. Also, Nesca et al. (2013) found that miR-34a was significantly upregulated in the serum of patients with T2DM

[50]. In addition, an increase in miR-34a is associated with the activation of p53, and results in sensitization to apoptosis and impaired nutrient-induced secretion [49,50]. On the other hand, sustained activation of NF-kB has been reported in retina cells of diabetic patients with its proinflammatory and proapoptotic consequences [51]. However, p53 accumulating under such conditions seems to be sequestered in the nucleolus, with reduced potential for transactivation of its target genes [52]. In fact, the levels of Bax, one of the mediators of p53-induced apoptosis were suppressed rather than increased [53]. Taken together, it could be hypothesized that in diabetic patients, with normal fundus show high level of expression of miR-34a due to hyperglycemia which is a p53-mediated effect as p53 is activated by increased oxidative stress. With disease progression there is a long lasting increased activation of NF-kB accompanied with suppression of the proapoptotic gene p53 with subsequent decline in the level of expression of p53-dependent miR-34a and reaches its minimum level in PDR diabetic patients. These data indicate the role of miR-34a in diagnosis and prognosis of DR.

Conclusions

Conclusively, we found that, diabetic patients showing increased serum level of only miR-146a without change in the levels of both miR-21 and miR-34a are in mild stage and they are candidates for moderate DR. A highly increased level of miR-146a as well as miR-21 with slight decrease in miR-34a the patient is in moderate stage and he is a candidate for severe NPDR. If serum level of both miR-146a and miR-21 are highly increased with marked suppression of miRNA-34a the patient is in stage of severe NPDR and this may lead to progression of the disease to be severe PDR. When the serum level of miR-34a is aggressively diminished along with very highly increased levels of both miR-146a and miR-21, the typical case of severe PDR is diagnosed. We provide evidence that circulating miRNAs are promising for DR and the pattern of their serum expression together may add a new avenue for staging of the severity of DR.

The strong points of the present study mainly include the possibility of helping diagnosis by non-invasive method depending on evaluation of circulating miRNAs. Also, this can give chance for further studies to indicate this role and for management by studying the effect of miRNAs inducers or inhibitors.

The weak points are the difficulties to find out the required number of diabetic patients on the same antidiabetic regimen and the inability to classify patients into males or females. In addition, further studies are recommended to prove the role of the tested miRNAs in either diagnosis or prognosis.

Conflicts of interest

None of the authors has any conflicts of interest regarding this study.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bj.2020.11.003.

REFERENCES

[1] Whiting DR, Guariguata L, Weil C, Shaw J. IDF diabetes atlas: global estimates of the prevalence of diabetes for 2011 and 2030. Diabetes Res Clin Pract 2011;94:311−21.

[2] Arab M. Diabetes mellitus in Egypt. World health statistics quarterly Rapport trimestriel de statistiques sanitaires mondiales 1992;45:334−7.

[3] Herman WH, Ali MA, Aubert RE, Engelgau MM, Kenny SJ, Gunter EW, et al. Diabetes mellitus in Egypt: risk factors and prevalence. Diabet Med 1995;12:1126−31.

[4] Macky TA, Khater N, Al-Zamil MA, El Fishawy H, Soliman MM. Epidemiology of diabetic retinopathy in Egypt: a hospital-based study. Ophthalmic Res 2011;45:73−8.

[5] Fong DS, Aiello L, Gardner TW, King GL, Blankenship G, Cavallerano JD, et al. Retinopathy in diabetes. Diabetes Care 2004;27:s84−7.

[6] Cai J, Boulton M. The pathogenesis of diabetic retinopathy: old concepts and new questions. Eye 2002;16:242−60.

[7] Oqurtsova K, da Rocha Fernandes JD, Huang Y, Linnenkamp U, Guariguata L, Cho NH, et al. Global estimates for the prevalence of diabetes for 2015 and 2040. Diabetes Res Clin Pract 2017;128:40−50.

[8] Pusparajah P, Lee LH, Abdul Kadir K. Molecular markers of diabetic retinopathy: potential screening tool of the future? Front Physiol 2016;7:200.

[9] Simó-Servat O, Hernández C, Simó R. Diabetic retinopathy in the context of patients with diabetes. Ophthalmic Res 2019;62:211−7.

[10] Pescador N, Pérez-Barba M, Ibarra JM, Corbatón A, Martínez-Larrad MT, Serrano-Ríos M. Serum circulating microRNA profiling for identification of potential type 2 diabetes and obesity biomarkers. PloS One 2013;8:e77251.

[11] Rong Y, Bao W, Shan Z, Liu J, Yu X, Xia S, et al. Increased microRNA-146a levels in plasma of patients with newly diagnosed type 2 diabetes mellitus. PloS One 2013;8:e73272.

[12] Banerjee J, Nema V, Dhas Y, Mishra N. Role of microRNAs in type 2 diabetes and associated vascular complications. Biochimie 2017;139:9−19.

[13] Ambros V. The functions of animal microRNAs. Nature 2004;431:350−5.

[14] Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell 2009;136:215−33.

[15] Mastropasqua R, Toto L, Cipollone F, Santovito D, Carpineto P, Mastropasqua L. Role of microRNAs in the modulation of diabetic retinopathy. Prog Retin Eye Res 2014;43:92−107.

[16] Chen JF, Murchison EP, Tang R, Callis TE, Tatsuguchi M, Deng Z, et al. Targeted deletion of Dicer in the heart leads to dilated cardiomyopathy and heart failure. Proc Natl Acad Sci USA 2008;105:2111−6.

[17] Kovacs B, Lumayag S, Cowan C, Xu S. MicroRNAs in early diabetic retinopathy in streptozotocin-induced diabetic rats. Invest Ophthalmol Vis Sci 2011;52:4402−9.

[18] He S, Hu XW, Wang D, Han LF, Zhang DC, Wei C. Accuracy of microRNAs for the diagnosis of hepatocellular carcinoma: a systematic review and meta-analysis. Clin Res Hepatol Gastroenterol 2016;40:405−17.

[19] McClelland AD, Kantharidis P. microRNA in the development of diabetic complications. Clin Sci 2014;126:95−110.

[20] Qing S, Yuan S, Yun C, Hui H, Mao P, Wen F, et al. Serum miRNA biomarkers serve as a fingerprint for proliferative diabetic retinopathy. Cell Physiol Biochem 2014;34:1733−40.

[21] Hermeking H. The miR-34 family in cancer and apoptosis. Cell Death Differ 2010;17:193−9.

[22] Slabáková E, Culig Z, Remšík J, Souček K. Alternative mechanisms of miR-34a regulation in cancer. Cell Death Dis 2017;8:e3100.

[23] Bernardo BC, Ooi JY, Matsumoto A, Tham YK, Singla S, Kiriazis H, et al. Sex differences in response to miRNA-34a therapy in mouse models of cardiac disease: identification of sex-, disease-and treatment-regulated miRNAs. J Physiol 2016;594:5959−74.

[24] Hou Q, Tang J, Wang Z, Wang C, Chen X, Hou L, et al. Inhibitory effect of microRNA-34a on retinal pigment

epithelial cell proliferation and migration. Invest Ophthalmol Vis Sci 2013;54:6481–8.

[25] American Diabetes Association. Diagnosis and classification of diabetes mellitus. Diabetes Care 2014;37:S81–90.

[26] Inzucchi SE, Bergenstal RM, Buse JB, Diamant M, Ferrannini E, Nauck M, et al. Management of hyperglycemia in type 2 diabetes, 2015: a patient-centered approach: update to a position statement of the American Diabetes Association and the European Association for the Study of Diabetes. Diabetes Care 2015;38:140–9.

[27] Sniderman AD, Blank D, Zakarian R, Bergeron J, Frohlich J. Triglycerides and small dense LDL: the twin Achilles heels of the Friedewald formula. Clin Biochem 2003;36:499–504.

[28] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. Methods 2001;25:402–8.

[29] Kramer CK, Rodrigues TC, Canani LH, Gross JL, Azevedo MJ. Diabetic retinopathy predicts all-cause mortality and cardiovascular events in both type 1 and 2 diabetes: meta-analysis of observational studies. Diabetes Care 2011;34:1238–44.

[30] Sena CM, Pereira AM, Seiça R. Endothelial dysfunction—a major mediator of diabetic vascular disease. Biochim Biophys Acta 2013;1832:2216–31.

[31] Joussen AM, Poulaki V, Le ML, Koizumi K, Esser C, Janicki H, et al. A central role for inflammation in the pathogenesis of diabetic retinopathy. FASEB J 2004;18:1450–2.

[32] Dasbach EJ, Fryback DG, Newcomb PA, Klein R, Klein BE. Cost-effectiveness of strategies for detecting diabetic retinopathy. Med Care 1991;29:20–39.

[33] Cortez MA, Bueso-Ramos C, Ferdin J, Lopez-Berestein G, Sood AK, Calin GA. MicroRNAs in body fluids—the mix of hormones and biomarkers. Nat Rev Clin Oncol 2011;8:467–77.

[34] Feng B, Chen S, McArthur K, Wu Y, Sen S, Ding Q, et al. miR-146a–mediated extracellular matrix protein production in chronic diabetes complications. Diabetes 2011;60:2975–84.

[35] Lee HW, Khan SQ, Khaliqdina S, Altintas MM, Grahammer F, Zhao JL, et al. Absence of miR-146a in podocytes increases risk of diabetic glomerulopathy via up-regulation of ErbB4 and Notch-1. J Biol Chem 2017;292:732–47.

[36] Yousefzadeh N, Alipour MR, Soufi FG. Deregulation of NF-κB–miR-146a negative feedback loop may be involved in the pathogenesis of diabetic neuropathy. J Physiol Biochem 2015;71:51–8.

[37] Kato M, Park JT, Natarajan R. MicroRNAs and the glomerulus. Exp Cell Res 2012;318:993–1000.

[38] Liang M, Liu Y, Mladinov D, Cowley AW Jr, Trivedi H, Fang Y, et al. MicroRNA: a new frontier in kidney and blood pressure research. Am J Physiol Renal Physiol 2009;297:F553–8.

[39] Tang J, Kern TS. Inflammation in diabetic retinopathy. Prog Retin Eye Res 2011;30:343–58.

[40] Tomić M, Ljubić S, Kaštelan S. The role of inflammation and endothelial dysfunction in the pathogenesis of diabetic retinopathy. Coll Antropol 2013;37:51–7.

[41] Usui-Ouchi A, Ouchi Y, Kiyokawa M, Sakuma T, Ito R, Ebihara N. Upregulation of Mir-21 levels in the vitreous humor is associated with development of proliferative vitreoretinal disease. PloS One 2016;11:e0158043.

[42] Chen Q, Qiu F, Zhou K, Matlock HG, Takahashi Y, Rajala RV, et al. Pathogenic role of microRNA-21 in diabetic retinopathy through downregulation of PPARα. Diabetes 2017;66:1671–82.

[43] Lai JY, Luo J, O'Connor C, Jing X, Nair V, Ju W, et al. MicroRNA-21 in glomerular injury. J Am Soc Nephrol 2015;26:805–16.

[44] Lin Y, Shen J, Li D, Ming J, Liu X, Zhang N, et al. MiR-34a contributes to diabetes-related cochlear hair cell apoptosis via SIRT1/HIF-1α signaling. Gen Comp Endocrinol 2017;246:63–70.

[45] Shen Y, Xu H, Pan X, Wu W, Wang H, Yan L, et al. miR-34a and miR-125b are upregulated in peripheral blood mononuclear cells from patients with type 2 diabetes mellitus. Exp Ther Med 2017;14:5589–96.

[46] Banerjee J, Khanna S, Bhattacharya A. MicroRNA regulation of oxidative stress. Oxid Med Cell Longev 2017;2017:2872156.

[47] Zhang H, Zhao Z, Pang X, Yang J, Yu H, Zhang Y, et al. MiR-34a/sirtuin-1/foxo3a is involved in genistein protecting against ox-LDL-induced oxidative damage in HUVECs. Toxicol Lett 2017;277:115–22.

[48] Thounaojam MC, Jadeja RN, Warren M, Powell FL, Raju R, Gutsaeva D, et al. MicroRNA-34a (miR-34a) mediates retinal endothelial cell premature senescence through mitochondrial dysfunction and loss of antioxidant activities. Antioxidants 2019;8:328.

[49] Lovis P, Roggli E, Laybutt DR, Gattesco S, Yang J-Y, Widmann C, et al. Alterations in microRNA expression contribute to fatty acid–induced pancreatic β-cell dysfunction. Diabetes 2008;57:2728–36.

[50] Nesca V, Guay C, Jacovetti C, Menoud V, Peyot ML, Laybutt DR, et al. Identification of particular groups of microRNAs that positively or negatively impact on beta cell function in obese models of type 2 diabetes. Diabetologia 2013;56:2203–12.

[51] Badrichani AZ, Stroka DM, Bilbao G, Curiel DT, Bach FH, Ferran C. Bcl-2 and Bcl-x L serve an anti-inflammatory function in endothelial cells through inhibition of NF-κB. J Clin Invest 1999;103:543–53.

[52] Klibanov SA, O'Hagan HM, Ljungman M. Accumulation of soluble and nucleolar-associated p53 proteins following cellular stress. J Cell Sci 2001;114:1867–73.

[53] Miyashita T, Reed JC. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell 1995;80:293–9.