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

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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.

Patients and 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 [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 (ARM) 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 (HbA1c) 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 the homogenized sample was incubated for 15 min at room temperature. 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 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 miScript reference primer, 2.5 μl specific primers for miR-146 (55501996. Willwoft Co., Birmingham, UK), miR-34a (55502000. Willwoft Co., Birmingham, UK) and miR-21 (55501997. Willwoft 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 Supplemental 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−ΔΔCt) [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−ΔΔCt). 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. 2].
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 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 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 can 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.

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 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 up-regulation [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

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].

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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 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]. Thou-nojam 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...
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.

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 anti-diabetic 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.

Authors' individual contribution

Conceptualization: Hend Gouda Helal, Mohammed H. Rashed, Ahmed Daifalla MD; Measurements: Hend Gouda Helal, Mohammed H. Rashed, Omnia Alsaiad Abdullah, Tamer Ibrahim Salem; Revision: Ahmed Daifalla MD, Tamer Ibrahim Salem, Omnia Alsaiad Abdullah; Writing: Hend Gouda Helal, Mohammed H. Rashed.

Conflict of interest

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

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Appendix A. Supplementary data

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

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Yousefzadeh N, Alipour MR, Soufi FG. Deregulation of NF-


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