Expression Level of Circulating miR-93 in Serum of Patients with Diabetic Nephropathy

Diyabetik Nefropati Hastalarının Serumlarında Serbest miR-93 Ekspresyon Düzeyleri

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Abstract

Objective: Diabetic nephropathy is a long-term complication of diabetes and is manifested as reduced glomerular filtration rate, increased urinary albumin excretion, and glomerular lesions. The study aim was to determine the expression level of serum cell-free mir-93 in diabetic patients with or without DN and compare that to healthy controls.

Material and Methods: In this case-control study, 61 type 2 diabetes patients (21 without diabetic nephropathy, 20 with microalbuminuria and 20 with macroalbuminuria), and 22 healthy controls were included. Cell-free microRNA was extracted from the serum of participants and real-time polymerase chain reaction was performed using SYBR Green master mix. The gene of hsa-miR-16 was used as a reference gene.

Results: Our findings revealed a significant downregulation of miR-93 expression in the serum of diabetic patients with or without nephropathy compared to the healthy individuals (p<0.005). However, there was no significant difference between the three groups of diabetic patients presenting different degrees of nephropathy.

Conclusion: Serum miR-93 is a good diagnostic marker for diabetes, but is not useful to distinguish between the diabetics with and without nephropathy.

Keywords: Diabetes; diabetic nephropathy; microRNA; miR-93

Introduction

Diabetes mellitus is a common chronic metabolic disorder with a considerable economic burden worldwide, particularly in low and middle-income countries (1, 2). The prevalence of diabetes in Iranian adults was estimated to be 8.5% by IDF (International Diabetes Federation) in 2015 (3). The number of people with type 2 diabetes mellitus (T2DM) was estimated as 415 million in 2015, and is expected to increase to 642 million by 2040 (3). The high economic burden of diabetes is likely related to its care and manage-
Diabetic nephropathy (DN) is the most serious microvascular and second most common complication of diabetes and is the leading cause of end-stage renal disease (ESRD) (11, 12). Several mediators and pathways, including hyperglycemia, advanced glycation end products (AGEs), protein kinase C, oxidative stress, inflammation, angiogenesis, renin-angiotensin–aldosterone system (RAAS), and the AGE/RAGE (advanced glycation end-products/receptor AGE) are involved in the pathogenesis and progression of DN (13, 14). In addition, some genetic factors may also predispose some patients to a higher risk of developing diabetes as well as its complications (15). Several studies have so far investigated the association between genetic variants of various genes such as ELMO1 and DN (16).

A growing body of evidence considers circulating microRNAs as potential biomarkers for early detection of various diseases. MicroRNAs are short non-coding RNAs (~21–24 nucleotides in length) that modulate physiological and pathological processes by posttranscriptional regulation of target genes of different pathways (17, 18). Dysregulation of miRNAs is correlated with pathogenesis of diabetes, and mainly influences the function of pancreatic β-cells, insulin resistance or both. In addition, the progression of diabetes is associated with distinct modifications in the profile of serum miRNAs (19). Various microRNAs have been implicated in DN pathogenesis (20), and the signature of circulating miRNAs can be used to predict disease progression (21). MiR-93 regulates microvascularization in the kidney and reduces angiogenesis by downregulating vascular endothelial growth factor (VEGF) (22, 23). The aim of this study was to determine the expression levels of serum miR-93 in diabetic patients with and without nephropathy and compare that to healthy controls.

Material and Methods

Patient characteristics

Sixty-one T2DM patients and 22 healthy controls were enrolled at the Diabetes Clinic, affiliated to Diabetes Research Centre (Shariati hospital, Tehran University of Medical Sciences) and Rast Ravesh Clinic (Alorz University of Medical Sciences) from August 2015 to September 2016 for a case-control study. T2DM diagnosis was confirmed by the ADA (American Diabetes Association) criteria (24) or history of using antidiabetic medications. The demographic information and disease history of each participant was recorded; parameters like weight, height, fasting blood sugar (FBS), HbA1c, serum urea and creatinine, and urine albumin/creatinine were measured. Glomerular filtration rate (GFR), body mass index (BMI) and albumin to creatinine ratio (ACR) were calculated based on the above, and persistent proteinuria was confirmed by the urinary albumin to creatinine ratio (ACR).

Patients with at least a 5-year history of diabetes without any evidence of nephropathy were selected as the control diabetes group. The remaining diabetic patients with nephropathy were stratified into the microalbuminuria (ACR: 30-299 µg/mg) and macroalbuminuria (ACR≥300 µg/mg) groups according to the KDOQI criteria (25). Healthy individuals older than 20 years of age, without any history or evidence of diabetes and nephropathy, and FBS less than 100 mg/dL were considered as the healthy control group. The exclusion criteria for all groups were pregnancy, lactation, history of smoking and alcohol consumption, obesity (BMI≥30), hypertension (systolic ≥140 or diastolic ≥90 or hypertension medication) and insulin therapy.

Informed consent was obtained from all participants before enrollment. The study protocol was approved by the Ethics Committee at Zanjan and Tehran University of Medical Sciences.

Sample collection and serum preparing

Blood samples were collected by Venoject in two tubes, one with and one without EDTA, for measuring HbA1c, and serum isolation and biochemical assays, respectively. To separate the serum, the blood samples were incubated for about an hour at room temperature and then centrifuged at 3000 g for 15 min. The clear supernatant was harvested and divided into two parts: one aliquot was used for biochemical assays and another was stored at -80°C in DNase/RNase free tube till use.

Biochemical measurements

Biochemical parameters including FBS and blood urea were measured using enzymatic assays. Serum creatinine was measured by Jaffe method using creatinine kit (Pars Azmun, Iran) and BIOLIS 24i Premium (Tokyo Boeki Machinery Ltd Japan). HbA1c was measured using HPLC with the Tosoh G8 instrument (South San Francisco, CA).
RNA extraction, cDNA synthesis, and real-time PCR

Frozen serum samples were thawed at room temperature and centrifuged at 3000 g for 5 min to pellet the cells, and cell-free microRNA was extracted from 200 µL of serum using miRCURY™ RNA Isolation Kits-Biofluids (Exiqon®, Denmark) according to the manufacturer’s instructions (www.exiqon.com). Any contaminating genomic DNA was eliminated by DNaseI. To detect any inhibitor and also the potential loss of RNA, the serum was spiked with synthetic RNAs (Exiqon, Denmark). The concentration of the extracted RNA was determined by NanoDrop 2000c spectrophotometer (Thermo Scientific, USA). The cDNAs were synthesized by Universal cDNA synthesis kit (Exiqon, Denmark) using 5–10 ng miRNA. Real-time PCR was carried out using LNA™ primers, ExiLENT SYBR Green master mix (Exiqon, Denmark) and 10x diluted cDNA on step one instrument (ABI, USA) according to the manufacturer’s instructions. The hsa-miR-16 and has-miR-93 were used as reference and candidate genes respectively. All experiments were performed in duplicate. PCR efficiencies were determined using LinRegPCR 12.x software (AMC, Amsterdam, http://LinRegPCR.nl).

Data Analysis

Real-time PCR data were analyzed by GenEX 5 (MultiD, Sweden) and SPSS (version 21 for Windows, SPSS Inc., USA) programs. All variables (BMI, FBS, HbA1c, serum urea, Cr, urine Alb/Cr ratio and GFR) were compared between the groups using Kruskal-Wallis or one-way ANOVA, as appropriate. For miR-93 gene expression analysis, the data were first adjusted by a spike in RNA, and the fold change in gene expression in the diabetic groups was normalized to the mean of the healthy control group using Pfaffl formula (26). The data were also adjusted for age, HbA1c, and duration of diabetes using the logistic regression model (each time for a covariate). Variables were compared between the groups using Kruskal-Wallis and also Mann-Whitney nonparametric test. A threshold value of 0.05 was considered as statistically significant. Receiver operating characteristics (ROC) curve was plotted to determine the correlation between miR-93 and the diabetic and non-diabetic state. Correlation of GFR (Glomerular Filtration Rate) and miR-93 expression was analyzed using Spearman’s correlation test.

Results

Mean age of participants was 57±10.5 years and 60.25% (50) of them were male. Clinical and biochemical characteristics of all participants in the four study groups (healthy controls, T2DM without nephropathy, T2DM with microalbuminuria and T2DM with macroalbuminuria) are summarized in Table 1. There was a significant difference in BMI, FBS, HbA1c, serum urea, serum Cr, urine Alb/Cr ratio and GFR between the study groups (Table 1).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal healthy control</th>
<th>Diabetic without nephropathy</th>
<th>Diabetic with microalbuminuria</th>
<th>Diabetic with macroalbuminuria</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>22</td>
<td>21</td>
<td>20</td>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>9/13</td>
<td>11/10</td>
<td>16/4</td>
<td>14/6</td>
<td>-</td>
</tr>
<tr>
<td>Age (years)</td>
<td>47.2±7.2</td>
<td>61.5±6.5</td>
<td>64.7±6.8</td>
<td>54.8±11.6</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Duration of diabetes (y)</td>
<td>N/A</td>
<td>12.9±4.8</td>
<td>15.3±4.8</td>
<td>13.1±6.5</td>
<td>0.33</td>
</tr>
<tr>
<td>BMI (Kg/m²)</td>
<td>24.88±1.18</td>
<td>23.20±1.8</td>
<td>23.94±1.26</td>
<td>26.25±2.67</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>FBS (mg/dL)</td>
<td>89.6±6.1</td>
<td>138.3±52.2</td>
<td>187.3±70.5</td>
<td>147±72</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.3±0.2</td>
<td>6.8±.09</td>
<td>7.7±1</td>
<td>8.5±2.1</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Serum urea (mg/dL)</td>
<td>25.9±5.6</td>
<td>37±9.8</td>
<td>45.7±13.8</td>
<td>60.1±30.2</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Serum Cr (mg/dL)</td>
<td>0.98±0.2</td>
<td>0.98±0.19</td>
<td>1.9±2.0</td>
<td>1.6±0.6</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Urine Alb/Cr</td>
<td>23±4.4</td>
<td>26.4±4.7</td>
<td>104±51</td>
<td>819±420</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>GFR (mL/min/1.73 m²)</td>
<td>83±13.8</td>
<td>72±11.3</td>
<td>67.6±16.6</td>
<td>55.2±28.8</td>
<td>0.002*</td>
</tr>
</tbody>
</table>

Values are means ± standard deviation.

GFR: Glomerular filtration rate; FBS: Fasting blood sugar; BMI: Body mass index; HbA1c: Hemoglobin A1c; Cr: creatinine.

*p<0.05 significant.
The extracted RNA was evaluated by NanoDrop, and the OD 260/280 was 2.7-2.2 and RNA concentration was 18±6.2 ng/µl. The mean CT value of miR-93 and miR-16 expression in the entire cohort was 31.72±2.6 and 24±1.5, respectively. Comparison between the diabetic patients (irrespective of kidney function) and the healthy controls showed a significant difference in miR-93 expression (p=0.005), but no significant difference was seen within the diabetic group (Figure 1A). The downregulation of miR-93 expression was observed in 68% of the diabetic patients without nephropathy, 88% of those with microalbuminuria and 72% of the patients with macroalbuminuria (Figure 1B). Furthermore, after adjusting for HbA1c and duration of diabetes, no significant association was seen between GFR (GFR<60 vs GFR≥60) and miR-93 expression among all diabetic patients. However, there was a significant but weak negative correlation between mir-93 expression level and Alb/Cr ratio (r=-0.31, p=0.005). Finally, as per the ROC results, low expression of miR-93 was an independent predictor of diabetes (AUC=0.72, p=0.004, 95% CI: 0.59-0.84) (Figure 2).

Discussion

Our results showed a significant decrease in the expression of mir-93 in T2DM patients compared to the healthy controls, while no significant difference was found among the diabetics with different renal functions. To the best of our knowledge, this is the first study that evaluates circulating cell-free miR-93 in the serum of patients with DN. The AUC (72%) indicated that although miR-93 can accurately discriminate between the diabetics and healthy individuals, it failed to discriminate between patients with DN from those diabetics without nephropathy. MicroRNAs have been considered as therapeutic targets and also potential biomarkers in complex human disorders such as diabetes and cancer (19, 27-29). Circulating miRNAs seem to have the potential of detecting the early development of diabetes several years before the symptoms start manifesting (21, 30). Other studies have reported a decreased expression of miR-93 in some diseases. Salas-Perez et al. showed a downregulation of miR-93 expression in the peripheral blood mononuclear cells (PBMCs) of T1DM patients (0.331±0.05, p<0.02) compared to healthy controls (31).

Long et al. analyzed the expression of miR-93 and VEGF in the glomeruli of diabetic db/db mice, and reported a significant downregulation of miR-93 expression in the diabetic mice compared to the db/m control mice. As VEGF has a critical role in microvascular complications of diabetes, they concluded that mir-93 may act as a key regulator of the VEGF gene (23). In another study, Saito et al. identified a negative correlation between miR-93 expression levels and VEGF-A and concluded that mir-93 probably plays a role in the pathogenesis of acute Kawasaki disease (32). Furthermore, Ulbing et al. reported a decreased expression of miR-93-5p in patients with chronic kidney disease (CKD) at stages 4 and 5 compared to healthy controls, indicating that miR-93 downregulation may drive the progression of DN to CKD (22). A recent study by Shawn et al. showed that in the presence of high glucose lev-
els, miR-93 contributes to chromatin remodeling in podocytes of kidney (33). According to their results, miR-93 had a significant function in angiogenesis and its dysregulation resulted in diabetic complications. The role of miR-93 in VEGF inhibition and angiogenesis has also been reported in other diseases, especially cancer. Some studies have reported an oncogenic role of miR-93, e.g., in gastric (34) and bladder cancers (35), while others have described a tumor suppressor activity of miR-93 in the breast (36), neuroblastoma (37) and colorectal (38) cancers. There are several limitations of our study, including the small number of patients in each study group, lack of mir-93 polymorphism analysis, and the expression of its target genes. Our results, therefore, need to be validated with studies on larger cohorts, along with the evaluation of downstream target gene expression.

Conclusion
The expression of miR-93 is influenced by the diabetic pathology and downregulated in diabetes both with and without accompanying nephropathy. Our results showed that miR-93 can discriminate between diabetes and normal glycemic status with high sensitivity, but not between the diabetic groups with varying degrees of nephropathy. As miRNAs are stable in plasma and serum samples, the detection of circulating miRNAs can help in the early prediction of certain diseases and enable suitable treatment strategies to be developed.

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Authorship Contributions
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