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Urinary miRNA-377 and miRNA-216a as biomarkers of nephropathy and subclinical atherosclerotic risk in pediatric patients with type 1 diabetes

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ABSTRACT

Background: Urinary microRNAs (miRNAs) play a role in the pathogenesis of chronic kidney disease (CKD).
Aim: To identify the expression of urinary miR-377 and miR-216a in 50 children and adolescents with type 1 diabetes (T1DM) compared with 50 healthy controls and assess their relation to the degree of albuminuria, glycemic control and carotid intimal thickness (CIMT) as an index of atherosclerosis.
Methods: Diabetic subjects were divided into normoalbuminuric and microalbuminuric groups according to urinary albumin creatinine ration (UACR). Urinary miRNAs were assessed using real time polymerase chain reaction. CIMT was measured using high resolution carotid ultrasound.
Results: The expression of urinary miR-377 was significantly higher in patients with microalbumiuria (median, 3.8) compared with 2.65 and 0.98 in normoalbuminic patients and healthy controls, respectively ($p < 0.05$). Urinary miR-216a was significantly lower in all patients with type 1 diabetes and the lowest levels were among the microalbumiuric group. Significant positive correlations were found between urinary miR-377 and HbA1C, UACR and CIMT while urinary miR-216a was negatively correlated to these variables.
Conclusions: Urinary miR-377 and miR-216a can be considered early biomarkers of nephropathy in pediatric type 1 diabetes. Their correlation with CIMT provides insights on the subclinical atherosclerotic process that occurs in diabetic nephropathy.

1. Introduction

Diabetic nephropathy (DN) is characterized by excessive accumulation of extracellular matrix (ECM) with thickening of glomerular and tubular basement membranes and increased amount of mesangial matrix, which ultimately progresses to glomerulosclerosis and tubulointerstitial fibrosis.[1,2] DN is a leading cause of end stage renal disease (ESRD).[3,4] Patients with type 1 diabetes mellitus (T1DM) are at risk of DN. Hence, a better understanding of the factors affecting disease from hyperfiltration to microalbuminuria, dipstick positive macroalbuminuria, impaired filtration and ESRD in patients with T1DM is urgently needed.[5,6]

Early identification of patients who are prone to develop renal complications would be an important step for their better management during the clinical course of this disease process.[7] There are no suitable biomarkers for the diagnosis of early stages of DN.[8] Albuminuria both reflects and results from nephropathy. Testing for albuminuria among people with diabetes identifies people at higher risk of subsequent complications and identifies people to whom to offer treatment.[9]

Elevated urinary albumin excretion (UAE) has been linked with increased cardiovascular disease mortality in individuals with and without diabetes.[10] Therefore, children with T1DM have the risk of cardiovascular diseases that may appear later. Thus, defining factors responsible for atherosclerosis is of great importance. The most significant changes in early subclinical period of atherosclerotic disease are endothelial dysfunction and increase in intima-media thickness observed in all arterial beds.[11] A noninvasive ultrasound measurement of carotid

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wall intima-media thickness (CIMT) is a marker of generalized atherosclerosis that predicts future cardiovascular events.[12,13]

Microalbuminuria has been the standard method for diagnosis of early stages of DN, however, its sensitivity and specificity for early disease detection are limited.[8] Not all patients with microalbuminuria progress to overt DN, nonalbuminuric DN is common and risk associated with microalbuminuria is elevated even at levels below currently accepted diagnostic thresholds.[14] It has also been shown that advanced structural alterations in the glomerular basement membrane may already have occurred by the time microalbuminuria becomes clinically evident.[15] There is therefore a need for alternative biomarkers allowing early identification of "at risk" individuals.[14]

In recent years, tremendous efforts are being made worldwide to delineate the role of microRNAs (miRNAs) in the pathogenesis of DN.[8] MiRNAs, a family of short (average of 22 nucleotides long), naturally occurring, small antisense non-coding RNAs have emerged as important post-transcriptional regulators of gene expression in many different health conditions.[16] A number of these miRNAs have also been identified in the extracellular environment. As they may regulate a significant portion of the transcriptome and proteome, considerable attention has focused on miRNAs as mediators or biomarkers of illness.[6,17]

The association between altered miRNA expression and the development and progression of the various diabetes complications implicates certain miRNAs in the development of diabetes-related injury in the heart, kidney, peripheral nerves, and retina.[18] A total of 27 miRNAs are present at significantly different levels in urine from patients at different stages of DN.[6] The expression of some miRNA in the urinary sediment correlated with proteinuria, renal function, and the degree of tubulointerstitial fibrosis.[19] miR-377 may play a critical role in the pathobiology of mesangial cells since they are known to undergo oxidative stress under high glucose ambience. MiR-216a is characteristically expressed in the pancreatic tissue. The relevance of miR-216a in chronic kidney diseases has also been described.[8,19,20]

Moreover, miRNAs are emerging as essential modulators of cardiovascular physiology and disease.[21] miRNA array data analysis indicates that miR-377 is a potential interest.[22] Both miR-377 and miR-216a may be implicated in endothelial dysfunction as well as the pathogenesis of cardiovascular disorders and atherosclerosis.[22–24] miR-377 may serve as a novel therapeutic target for stem cell-based treatment of ischemic heart disease.[23]

Hyperglycemia and increased oxidative stress play pivotal roles in the early stages of atherogenesis in diabetes, including impairment of endothelial function.[25] Thus, the aim of this study was to determine urinary miR-377 and miR-216a expression in children and adolescents with T1DM as potential early markers for DN and assess their relation to degree of albuminuria and glycemic control. Furthermore, we investigated CIMT in patients with T1DM without manifest micro- or macrovascular disease and its relation to the studied urinary miRNAs to explore their role in subclinical atherosclerosis among patients with T1DM.

2. Materials and methods

This cross-sectional study performed on 50 patients with T1DM diagnosed according to recommendations of American Diabetes Association.[26] Patients were recruited from Pediatric Diabetes Clinic, of Pediatric Hospital, Ain Shams University. The studied patients with T1DM were 27 males and 23 female patients and their age ranged from 6 to 18 years with a mean of 13.7 ± 3.3 years. Inclusion criteria were children and adolescents with type 1 diabetes ≤ 18 years with at least 5 years disease duration and on regular clinic visits and regular insulin therapy. We only enrolled diabetic patients without manifestations of renal disease by history and clinical examination. Patients with any clinical evidence of infection, hematological disorders, malignancy, hepatic dysfunction, urinary tract infections or disorders, congenital renal abnormalities, nephrotic syndrome, connective tissue disease or other autoimmune disorders and any other conditions that could influence C-reactive protein were excluded from the study. Moreover, microvascular complications (neuropathy and retinopathy) and macrovascular diabetic complications were also excluded to avoid any confounding factors that may affect miRNAs results. None of our studied patients received lipid-lowering drugs including statins or angiotensin converting enzyme inhibitors (ACE-Is). Another group of 50 age-, sex- and pubertal stage-matched healthy volunteers (30 males and 20 females with mean age 14.2 ± 2.1 years) were enrolled as controls. An informed consent was obtained from each patient or control subject or their legal guardians before enrollment into the study. The study was approved by the local ethical committee of Ain Shams University.

2.1. Routine clinical assessment

Clinical evaluation of the patients was based on detailed medical history from the parents, reviewing follow-up sheets and thorough clinical examination. All patients were subjected to detailed history with description of age of onset of diabetes, disease duration, insulin therapy and chronic diabetic complications (nephropathy, peripheral neuropathy, retinopathy or cardiovascular ischemic events). All patients were on insulin therapy using human insulin with a mean dose of 1.6 ± 0.27 IU/kg/day. Anthropometric measurements were recorded and body mass index (BMI) was calculated. Pubertal stage was determined according to Tanner's classification.[27] Blood pressure was measured after a 5-min rest in the seated position using mercury sphygmomanometer. If it was greater than 90th percentile for age and sex, the blood pressure was repeated twice for the validity of the reading. Fundus examination was performed through dilated pupils using 90-diopter Volk lens and biomicroscope for exclusion of diabetic retinopathy. The simple rapid bedside neuropathy disability score (NDS) was used as a screening tool for exclusion of neuropathy.[28]

2.2. Blood sampling and laboratory investigations

Peripheral blood (PB) samples were collected on ethylene diamine tetra-acetic acid (EDTA) (1.2 mg/mL) for analysis of HbA1c. Serum obtained from clotted samples by centrifugation for 15 min at 1000*g* was used for chemical analysis. Urine samples were collected for assessment of urinary albumin excretion and glomerular filtration rate (GFR) as well as isolation of microRNA.

Liver and kidney function tests, fasting lipid profile, as well as fasting and random blood glucose levels were measured using Cobas Integra 800 (Roche Diagnostics, Mannheim, Germany). Fasting blood glucose (FBG) was assessed during regular clinic visits with calculations of the mean values during the last 6 months prior to the study including the level of a blood sample at the start of the study. Dyslipidemia was defined based on percentiles according to age and sex following the report of the American Academy of Pediatrics (AAP)[29] and considered if at least one of the following was present; serum total cholesterol ≥ 200 mg/dL, low-density lipoprotein (LDL) cholesterol ≥ 100 mg/dL, high-density lipoprotein cholesterol (HDL) < 40 mg/dL, or serum triglyceride ≥ 150 mg/dL.[29] Further analysis was done after controlling for age and pubertal stage to avoid differences in lipid values.[30] Assessment of mean HbA1c% in the year preceding the study was performed using D-10 (BioRad, France). GFR was calculated by the Cockgroft-Gault equation.[31] Urinary albumin excretion (as an indicator of nephropathy) was measured in an early morning urine sample as albumin-to-creatinine ratio by an immunonephelometric method. Microalbuminuria and macroalbuminuria were present if urinary albumin excretion in at least 2 out of 3 consecutive urine samples over a 3- to 6-months period was 30–299 mg/g creatinine and ≥ 300 mg/g creatinine, respectively.[32,33] Potential factors affecting urinary albumin excretion were excluded.[34] Assessment of urinary miR-377 and miR-216a was performed as previously described.[6,19]

2.3. RNA isolation

The RNA from urine was isolated using the miRNeasy kit (Qiagen, GmbH, Hilden, Germany). In brief, 700 mL of QIAzol reagent was

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