E-CADHERIN-A SURROGATE MARKER AND ITS RELATION TO OTHER BIOMARKERS IN EARLY STAGES OF DIABETIC NEPHROPATHY IN EASTERN PROVINCE OF SAUDI ARABIAN POPULATION

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Abstract: Background: At present, it is very arduous to reliably envisage which, and when, diabetic patients will develop nephropathy and progress to kidney failure. Therefore, preventing diabetic nephropathy or delaying the disease progression by searching for novel biomarkers is very important. Objective: Determination of diagnostic and prognostic significance of soluble E-cadherin (sE-cadherin) and cystatin C as novel biomarkers of diabetic nephropathy in type 2 diabetic (T2D) Saudi patients. Patients and Methods: Type 2 diabetic patients (n=50) with urinary albumin excretions (UAE) > 300 mg/day (n=16) macroalbuminuria, between 30-300 mg/day (n=14) microalbuminuria and without urinary albumin excretions (UAE < 30 mg/24 h) (n=20) normoalbuminuria and 15 controls were enrolled in the study. sE-cadherin, Serum Cystatin C, beta2-microglobulin, urinary microalbumin levels, and creatinine clearances were determined in all groups. Results: There was significant increase in the values of fasting blood sugar(FBS), serum creatinine, NAG, b-microglobulin, Hb1Ac in different groups. There was no significant change of Hb1Ac and serum creatinine between diabetic mellitus (DM) with normalbuminuria and diabetic nephropathy microalbuminuria. The diabetic control and DM group presented with no significant change in E-cadherin (p=ns), however there was significant change of E-cadherin between DM with normalbuminuria and diabetic nephropathy with microalbuminuria (p<0.001) and diabetic nephropathy with macroalbuminuria. Serum creatinine, FBS, cystatin C and Hb1Ac were known predictors of E-cadherin as extracted from regression model [Y(E-cadherin)= -1792.2+ 0.51(S.Creatinine)+0.47 (FBS)+0.30(Cystatin C)+0.24(Hb1Ac)]. Cystatin C was significantly increased in DM as compared to diabetic control (p<0.001), however there was no change between DM normoalbuminuria and diabetic nephropathy macroalbuminuria (p=ns). Further there was significant increase in the values of Cystatin C in diabetic nephropathy with macroalbuminuria as compared to diabetic nephropathy with microalbuminuria and normalbuminuria. Linear regression model of cystatin showed only E-cadherin and b-microglobulin as independent variables [Y (cystatin C) = 0.301+0.559(E-cadherin) + 0.363(b-microglobulin)]. Conclusion: In conclusion, this study demonstrated that E-cadherin and cystatin C might play an important role in the development of early diabetic nephropathy and their measurement might become a useful and noninvasive marker than creatinine or 62-MG for early incipient diabetic nephropathy as well as for the evaluation of renal involvement of T2D patients.

Keywords: Cystatin C, β2-microglobulin, E-cadherin, Diabetic nephropathy.

BACKGROUND

The global prevalence of diabetes is expected to increase from 4% in 1995 to 5.4% by the year 2025 [1]. Diabetic nephropathy (DN), an important complication of diabetes mellitus (DM), is the most common cause of end-stage renal failure [2,3]. Recognized as a major cause of the end-stage renal disease followed by premature morbidity and mortality, is characterized by albuminuria, subsequent proteinuria, declining glomerular filtration rate and elevated blood pressure. Identification of risk at the earliest stages of diabetic nephropathy and identification of the pathological processes that lead to early loss of glomerular filtration rate (GFR) may permit the development and implementation of interventions at a stage when they are most likely to be effective.

Diabetic complications include macrovascular and microvascular disorders. Macrovascular disorders are coronary artery disease, cerebrovascular disease, and peripheral vascular disease; and microvascular disorders are nephropathy, retinopathy, and neuropathy [4]. Renal damage is an important and serious diabetic microvascular complication, and is the leading cause of end-stage renal disease [5]. Consequently, early diagnosis of nephropathy is very critical [6]. Microalbuminuria, which affects up to 40% of patients with diabetes, is traditionally considered a predictor of diabetic nephropathy (DN). The gold standard for measuring urine albumin excretion is still 24-hr urine collection. However, the
standard clearance technique necessitates timed urine collection, which is not only time-consuming but also subject to error. A random spot urine sample using albumin: creatinine ratio measurement (ACR) (American Diabetes Association, 2012; KDOQI (Kidney Disease Outcomes Quality Initiative) Guidelines, 2007) has been recommended for the diagnosis of microalbuminuria (American Diabetes Association, 2012; Gross et al., 2005; KDOQI (Kidney Disease Outcomes Quality Initiative) Guidelines, 2007). UAC in a random urine specimen may be an alternative for the diagnosis of microalbuminuria in diabetic patients, especially considering its low cost and accuracy.

But these markers are insensitive, unreliable, nonspecific, and there is a time delay between renal injury and detection [7]. Also, serum creatinine has been widely used as a marker of GFR, but it is not sensitive enough to detect decreased renal function. Therefore, the use of endogenous markers is also important in the evaluation of diabetic nephropathy. Thus, if other biomarkers are found with improved specificity and sensitivity, this could reverse or prevent the onset of renal damage.

Among these markers, previous studies demonstrated that serum cystatin C (cys C) might be a superior marker for the evaluation of renal function than serum creatinine [8]. However, the effectiveness of cystatin C for estimating GFR has not been sufficiently demonstrated in Saudi population with diabetes.

The aim of this study was to investigate the clinical usefulness of measuring E-cadherin and cystatin C levels as biomarker for detecting early stages of diabetic nephropathy and correlate the same with other parameters in assessing renal function such as 82-microglobulin (low molecular mass protein markers of glomerular filtration rate-GFR), creatinine (marker of GFR) and urinary N-acetyl-b-D-glucosaminidase (NAG) excretion (marker of glomerular and tubular dysfunction) in 50 type 2 (non-insulin-dependent) diabetic patients.

**STUDY DESIGN AND PARTICIPANTS**

**Subject Group**

Age matched type 2 diabetic patients (n =50) and diabetic control (n = 15) were initially enrolled in this study at King Khalid Hospital, Majmaah (Saudi Arabia) using the stringent inclusion and exclusion criteria recommended by the American Diabetes Association for type 2 diabetes between September 2011 and March 2012.

Twenty healthy age-matched subjects served as non-diabetic control. They were enrolled in this study if they had plasma glucose levels of <100 mg/dl after an overnight fasting, an eGFR of ≥60 ml/min/1.73 m2, and no history of diabetes and renal and cardiovascular diseases including hypertension and dyslipidemia.

**Inclusion Criteria**

All patients fulfilled the following inclusion criteria: age ≥18 years, eGFR ≥60 ml/min/1.73m2, and serum creatinine <1.2 mg/dl, stable renal function status without twofold elevations in serum creatinine for at least 5 preceding months.

**Exclusion Criteria**

We excluded the patients based on the following criteria: abnormal urinary sediment and active urinary tract infection; history of advanced chronic renal disease, defined as estimated glomerular filtration rate (eGFR) <30 mL/min/1.73 m2; severely uncontrolled diabetes, defined as hemoglobin A1c (HbA1c) level 10%; uncontrolled thyroid disorders, severe liver dysfunction, pregnancy, recent (within 6 months) history of acute myocardial infarction, stroke, and occlusive peripheral vascular disease.

**Ethics Statement**

This study was approved by the institutional review board at the Majmaah University. All subjects were informed about the purpose of the investigation and gave their written consent.

**Chemicals**

All chemicals were of the highest quality available. N-acetyl-b-D-glucosamine (NAG)
was obtained from Sigma Chemical Co. (St. Louis, MO) and Merck GmbH (Darmstadt, Germany).

Sample Collection
Heparinized blood samples were obtained from fasting subjects of all the groups. Blood samples were drawn without anticoagulant using vacutainer Advance Tubes (BD, Franklin Lakes, NJ) and kept at room temperature for 30 min. The serum was obtained by centrifugation (10 min, 3,000g), aliquoted and stored at 800C. All subjects (control and diabetic individuals) provided urine containers to store 10-20 ml second voided clean-catch urine samples in the early morning after an overnight fast. Plasma, hemolysates and urine samples were immediately frozen and stored at -80°C to prevent protein degradation.

Determination of Biochemical Parameters
For definition of the glycemic condition of the patients, fasting blood glucose in plasma (short-term control) and concentration of Glycosylated hemoglobin (HbA1c) hemolysates (long-term control) were determined. Blood glucose was measured by the commercial test using glucose oxidase (Analco, Poland). HbA1c levels were measured by commercially available immunoturbidimetric assay kits (Roche Diagnostic GmbH, Germany) on an automatic analyzer (Bayer Diagnostics). HbA1c in nondiabetic, good glycemic control, and poor glycemic control were set to be <6%, <7%, and <8%, respectively.

Urinary microalbumin measurements were made on automatic analyzer (Hitachi Tokyo, Japan) by using commercial available immunoturbidimetric assay kits (Roche Diagnostics GmbH, Germany) and were evaluated on the basis of at least three consecutive measurements in 24 h. [9]. For simultaneous determination of creatinine in serum and urine the routine Jaffe method was used [10] on automatic analyzer (Bayer Diagnostics). Total urinary albumin concentrations were adjusted for creatinine concentration and expressed as urinary albumin protein/creatinine ratio (UACR), calculated as protein (g l–1) x 10 000 divided by creatinine (mmol l–1) with units of 10 g mmol–1 to be in accordance with previous studies [11,12]. Collections and values of UACR <30 mg/day was accepted as normoalbuminuric, between 30-300 mg/day were accepted as microalbuminuric and >300 mg/day as macroalbuminuric.

Urine Activity of NAG
was estimated by using colorimetric assay method in an analyzer (Roche Cobas Mira) using 4-methylumbelliferyl-N-acetyl-b-D-glucosaminide as substrate.

Estimation of Cystatin C and β2-Microglobulin in Plasma
Plasma concentrations of cystatin C and β2-MG were measured using latex particle-enhanced turbidimetry (PET) using a commercial kit (Dako, Denmark) on the automatic analyzer (Boehringer Mannheim GmbH, Mannheim, Germany).

ELISA Analysis for E-Cadherin in Urine
The concentration of soluble E-cadherin in urine samples was measured with an ELISA kit (Zymed Laboratories Inc, USA) using HEC-D-1 as the E-cadherin-specific antibody. The standard curve was created using the suppliers’ lyophilized human E-cadherin. And the assay was performed according to the manufacturer’s specifications as described previously [13]. E-cadherin concentrations were adjusted for creatinine concentration and expressed as E-cadherin/creatinine index calculated as protein (g l–1) x 10 000 divided by creatinine (mmol l–1) with units of 10 g mmol–1 to be in accordance with previous studies [11,12].

Statistical Analysis
The data was entered and analyzed using SPSS 20.0. Mean ± S.D was given for quantitative variables like age, and biochemical parameters etc. One-way ANOVA was applied to compare the means of biochemical parameters among groups. Post-hoc Tukey test was applied to observe which group means differ. Pearson correlation was applied to observe correlations between quantitative variables. Linear regression analysis was applied to find predictors for Cystatin C and E-cadherin markers. A p-value of <0.05 was considered as statistically significant.

RESULTS
Based on urinary albumin/creatinine ratio

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(UACR), the type 2 diabetic patients were categorized into three groups, Normoalbuminuric or DM group without nephropathy & albuminuria (UACR<30 mg/g, n = 20), Microalbuminuria or Early DN (Diabetic nephropathy) group (30 < UACR < 300 mg/g, n = 16); Macroalbuminuria or overt DN (Diabetic nephropathy) group (UACR≥300 mg/g, n = 14).

**Determination of Biochemical Parameters**

No significant differences were determined in gender and age between groups (Table 1). A statistically significant increase in diabetic patients was found in fasting blood glucose, glycosylated hemoglobin, urinary NAG, α and β macroglobulin compared with normal control. Glycosylated hemoglobin was measured in samples obtained from different study groups as shown in Table 1. The level of HbA1c was higher in patients with microalbuminuria as compared to normoalbuminuria controls (p<0.05). Further HbA1c was higher in patients with macroalbuminuria compared to patients with microalbuminuria (p<0.01).

The urinary NAG ((units/mmol creatinine) levels were significantly elevated in patients (Table 1) with microalbuminuria (1.69±0.90) and macroalbuminuria (6.98±3.91) as compared with patients with normoalbuminuria (1.55±0.70) and control subjects (1.10±0.50).

Table 1: Demographic profile of diabetic patients and control groups. All data are presented as mean±SD. A P value of 0.05 denotes the presence of a statistically significant difference.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normal Control group</th>
<th>Type 2 Diabetic Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DM group UACR&lt;30 Normoalbuminuria</td>
<td>DN1 group 30 &lt; UACR &lt; 300 Microalbuminuria</td>
</tr>
<tr>
<td>Total Numbers (n)</td>
<td>15</td>
<td>20</td>
</tr>
<tr>
<td>Male</td>
<td>8</td>
<td>11</td>
</tr>
<tr>
<td>Female</td>
<td>7</td>
<td>9</td>
</tr>
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<td>Age Range (years)</td>
<td>41-51</td>
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<tr>
<td>Mean Age (years)</td>
<td>46±3</td>
<td>50±3</td>
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<tr>
<td>Fasting blood sugar (FBS) (mmol/l)</td>
<td>4.71±0.41</td>
<td>8.09±1.01</td>
</tr>
<tr>
<td>Serum creatinine (mg/dl)</td>
<td>0.65±0.13</td>
<td>0.81±0.11</td>
</tr>
<tr>
<td>NAG (units/mmol creatinine)</td>
<td>1.10±0.50</td>
<td>1.55±0.70</td>
</tr>
<tr>
<td>Beta-2 microglobulin (ug/mmol creatinine)</td>
<td>1.42±0.28</td>
<td>2.13±0.67</td>
</tr>
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</table>
Estimation of Creatinine in Plasma
Creatinine-based estimation of GFR was significantly higher (p<0.01) in patients with normoalbuminuria (0.81±1.11 umol/l) as compared to normal diabetic control group (0.65±0.13 umol/l). The level of creatinine in patients with microalbuminuria (0.86±0.11 umol/l) was slightly higher as compared with normoalbuminuria (0.81±1.11 umol/l) but not significantly. Further there was significant difference (p<0.05) in the creatinine levels among the patients with microalbuminuria (0.81±1.11 umol/l) as compared to patients with macroalbuminuria (1.76±0.26 umol/l).

Estimation of Cystatin C in Plasma
Cystatin-based estimation of GFR was significantly higher in diabetic patients with normoalbuminuria (0.93±0.15 mg/L) compared with normal control group (0.56±0.19 mg/L). Further the level of cystatin was significantly higher in patients with macroalbuminuria (1.84±0.41 mg/L) as compared to microalbuminuric patients (1.12±0.20 mg/L).

ELISA Analysis of Urinary Soluble E-Cadherin
To explore the changes of urinary level of sE-cadherin in DN patients, we performed ELISA analysis on 60 urine samples from DM (n = 20), DN1 (n = 16), DN2 (n = 14) and control groups (n = 15). The clinical characteristics of the subjects were shown in Table 1. To avoid the effect of urine volume, urinary levels of sE-cadherin were presented after correction for urinary creatinine concentration (sEcadherin/Cr) as shown in Table 3. The ELISA data also demonstrated that urinary sE-cadherin-to-creatine ratio was significantly increased in DN1 and DN2 groups versus DM or control group (2652.89 ± 114.3 and 5634.39 ± 306.73 vs 767.83 ± 13.01 or 660.92 ± 8.05 μg/g; p < 0.001), and markedly elevated in DN2 group versus DN1 group (5634.39 ± 306.73 vs 767.83 ± 13.01 μg/g; p = <0.001). But no significant difference of urinary sEcadherin/Cr was found between DM group and control group (717.83 ± 13.01 vs 660.92 ± 8.05 μg/g; p = ns).

Figure 1: Glycosalated Hemoglobin (HbA1c) levels in normal control and in type 3 diabetes mellitus. All data are presented as mean±SD.

Figure 2: Graphical representation of serum creatinine in different groups.
DISCUSSION

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction and failure of
various organs, especially the eyes, kidneys, nerves, heart and blood vessels. Type 2 diabetes mellitus has quickly become a global health problem due to rapidly increasing population growth, aging, urbanization and increasing prevalence of obesity and physical inactivity. There is, therefore, an urgent need to prevent diabetes and its complications [14].

Diabetic nephropathy is a major microvascular complication and a leading cause of chronic kidney failure in individuals with both type 1 and type 2 diabetes. The risk is dramatically elevated with poor blood glucose control and the greatest rate of progression occurs with elevated blood pressure, confirming that hemodynamic and metabolic factors participate in the development and progression of this disorder. The glomerulus is considered to be the primary site of initial renal injury, but increasing evidence points to the tubulointerstitium also playing a critical role via the process of epithelial-mesenchymal transition (EMT) [15]. During this process, epithelial cells acquire features of mesenchymal cells such as myofibroblasts, resulting in loss of E-cadherin expression, the acquisition of mesenchymal markers such as α-smooth muscle actin (α-SMA), and the increased deposition of extracellular matrix (ECM).

NGAL, a 25 kDa protein belonging to the lipocalin family, is hyperproduced in the kidney tubules within a few hours after deleterious stimuli and is regarded as an excellent early predictor of acute renal damage [16]. NAG is a 140 kDa lysosomal brush border enzyme found in the proximal tubular cells [17,18]. Urinary NAG, elevated in acute and chronic kidney disease, is of diagnostic value for the early detection of acute kidney injury [18,19].

Searching for novel biomarkers can be done using tissues and/or biofluids. The urine is an ideal biofluid for biomarker discovery in kidney diseases and diabetes mellitus. Urine samples obtained from patients with other diseases or disorders that have clinical, biochemical and metabolic profiles similar to those of the disease of interest must be included as the other controls. Finally, a single ideal biomarker may not exist for each disease. Therefore, evaluating a panel of multiple biomarkers may be required.

Human cystatin C, low molecular weight (13 kDa) is produced at a constant rate by all body tissues and is freely filtered mainly from circulating blood by renal glomerular filtration, followed by tubular reabsorption and final degradation. The low molecular mass of cystatin C, in combination with its stable production rate, indicates that the plasma concentration of cystatin C is almost exclusively determined by the glomerular filtration rate (GFR), what makes cystatin C an excellent indicator of GFR [20-22]. According to TL AN et al combined measurements of cystatin C in serum and urine may be useful, especially to detect mild reduction of GFR and renal tubular damage, but in the case of urine a sensitive ELISA test should be used [23]. In addition, because the amount of Cys-C excreted in the urine was less, its clinical usefulness was not evident. Nonetheless, recently, its role as a marker of early renal tubular dysfunction has been revealed, [24] and particularly, it is useful to the early detection of renal dysfunction in diseases anticipated to progress to chronic renal failure such as diabetic nephropathy [25-27].

E-cadherin is one of the most important molecules in cell-cell adhesion in epithelial tissues. It is localized on the surfaces of epithelial cells in regions of cell-cell contact known as adherens junctions [28]. Previous studies have revealed that loss function of E-cadherin contributes to progression in cancer by increasing proliferation, invasion and/or metastasis [29,30]. Additionally, only a limited number of researchers discovered that urinary soluble E-cadherin was increased in bladder cancers [31,32] and serum soluble E-cadherin was a prognostic marker in various malignancies [13,34]. But, the relationship between E-cadherin and diabetic nephropathy had seldom been reported. In the present study, all subjects were selected by strict application of inclusive and exclusive criteria, to avoid the influence of tumour and other factors on the results. There was no significant difference in the values of E-cadherin between diabetes mellitus and normal control group. However, the E-cadherin level was increased 4 times
in DN1 group and 8.5 times in DN2 group versus control group.

This makes clear that the level of urinary sEcadherin was higher in DN patients, and increased with the development of disease, but the reasons for the increase are still unclear. As we all know, with the development of renal damage of DN, ischemia and apoptosis of renal tubular epithelial cells will increase [35,36]. Previous studies had demonstrated that ischemia and apoptosis could lead to degradation and cleavage of E-cadherin by proteolytic enzyme activation [37,38]. Thus, the degradation and cleavage induced by renal damage may be a major reason for the increase of urinary sE-cadherin.

Nevertheless, to the best of our knowledge, the present study is the first to simultaneously check both serum cystatin C and E-cadherin in patients with diabetic nephropathy, and might be a more accurate, convenient, and effective indicator for the detection of renal function. In summary, our present study proves urinary sE-cadherin has a potential clinical diagnostic value for diabetic nephropathy in Type 2 diabetes mellitus patients. Large-scale and longitudinal follow-up studies are required in future to elucidate the pathogenic mechanism of urinary sE-cadherin and to evaluate whole spectrum of diabetic nephropathy including patients with a moderate to severe decrease of GFR.

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