Urinary heme oxygenase-1 as a new and early marker of diabetic nephropathy

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Introduction
Microalbuminuria is considered an early marker of glomerular injury in patients with diabetes, but tubular injury can precede glomerular injury and cannot be detected by microalbumin. We need a new and early marker of kidney injury in normoalbuminuric diabetic patients. Urinary heme oxygenase-1 (uHO-1) is an enzyme that is produced in tubules in response to oxidative stress and can be a marker of tubular injury.
Aim
To investigate the clinical implication of uHO-1 as an early diagnostic marker in diabetic nephropathy (DN).
Patients and methods
A total of 65 diabetic patients, comprising 20 microalbuminuric patients and 45 normoalbuminuric patients, and 20 healthy participants as a control group were included in this study. Level of uHO-1 was detected by enzyme-linked immunosorbent assay.
Results
uHO-1 was highly significantly elevated ($P<0.000$) in microalbuminuric group compared with normoalbuminuric group and control group, respectively. Normoalbuminuric group was highly significantly elevated ($P<0.000$) in uHO-1 compared with the control group. uHO-1 was positively correlated with albumin–creatinine ratio but was not correlated with estimated glomerular filtration rate. Receiver operating characteristic curve analysis of uHO-1 levels for early diagnosis of DN revealed that the cutoff value of uHO-1 was 5.8 ng/ml (sensitivity 80% and specificity 71%) for early diagnosis of DN.
Conclusion
The findings of this study indicate that elevated levels of uHO-1 can be detected in normoalbuminuric diabetic patients and revealed renal tubular damage. uHO-1 may be used as an early biomarker for DN.

Keywords:
diabetic nephropathy, heme oxygenase-1, oxidative stress, renal tubular injury

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Introduction

Diabetic nephropathy (DN) is currently the leading cause of end-stage renal disease in the western world. Approximately 45% of the individuals on renal replacement therapy worldwide are diabetic; thus, diabetes mellitus is the principal cause of renal replacement therapy [1].

DN is characterized by several changes in the glomeruli and tubules, which include basement membrane thickening, glomerular and tubular hypertrophy, glomerulosclerosis, and tubulointerstitial fibrosis. The classification of the DN stages is based mainly on glomerular changes, but now the attention increases toward tubules that play an important role in the pathogenesis of DN. Urinary tubular proteins may precede microalbuminuria, suggesting that tubular affection occurs early in the course of the disease not secondary to glomerular damage, and tubulointerstitial damage has an important role in the pathogenesis of early DN [2,3].

Unless microalbuminuria is a diagnostic marker for DN, a Japanese study had shown that histopathologic changes of DN may occur in patients with normoalbuminuric, which means urinary albumin may not be enough to diagnose patients with early DN [4].

Recently, many markers of inflammation and tubular injury have been identified as forceful predictors of renal damage in patients with diabetes like tumor necrosis factor receptor 1 and 2 [5]. These markers

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suggest that inflammation and tubular damage occurs very early in the disease before glomerular damage [2].

Oxidative stress-induced hyperglycemia is considered a key step in the pathogenesis of renal microvascular complications. Hyperglycemia causes antioxidative dysfunction in the cells and increases the reactive oxygen species, which leads to cellular oxidative stress [6]. At present, markers of inflammatory and oxidative processes accompanying tubular damage in DN are being assessed in the urine, like urinary heme oxygenase-1 (uHO-1) [7].

Heme oxygenase-1 (HO-1) is an isoenzyme of HO involved in heme degradation. It converts heme into carbon monoxide, iron, and biliverdin, which is converted to bilirubin by biliverdin reductase and is produced in response to oxidative stress [8]. HO-1 protects the tissues from vasoconstriction and vascular injury, inflammation, immune injury, oxidative stress, and cell damage. These properties of HO are derived, at least in part, from its products, such as carbon monoxide and bile pigments [9].

HO-1 is upregulated in proximal tubule cells in response to oxidant stress, so it can be an important biomarker of intrarenal activity and renal injury [10]. Recent studies showed that HO-1 within the damaged cells falls away into the cavity of renal tubules in patients with various kidney diseases [11].

The aim of this study was to investigate the diagnostic role of uHO-1 in early stages of DN and its correlation with the microalbumin in diabetic patients.

Patients and methods

This was a cross-sectional comparative study on 65 diabetic patients from outpatient clinic of Assuit University Hospital and 20 healthy control patients. All patients provided a written consent to participate in this study.

Group 1 included 20 diabetic patients with microalbuminuria, group 2 included 45 normo-albuminuric diabetic patients, whereas group 3 consisted of 20 age-matched and sex-matched nondiabetic control patients without any renal disease.

Exclusion criteria

Patients with history of diabetic ketoacidosis or hypoglycemic coma in the past 3 months preceding the study, urinary system disorders (macroscopic hematuria, abnormal sediment, urinary tract infection, tumors, glomerulonephritis, nephrolithiasis, or renal diseases other than DN), liver diseases, and cardiovascular diseases were excluded.

Blood and urine samples

Blood samples

Overall, 6 ml of blood was withdrawn after 12–14 h fasting and was divided into the following: 2 ml of blood in EDTA tube, for glycosylated hemoglobin, and 4 ml of blood in a plain vacutainer. Serum was separated for analysis of fasting glucose, urea, creatinine, and complete lipogram.

Urine samples

Fresh urine samples were collected from patients and control for detection of the urinary albumin and creatinine concentration. Fasting urine samples were collected in nonheparinized tubes and centrifuged at 1000*g* at 4°C for 5 min within 1 h of collection. Then, the supernatants were discarded. Pellets were washed twice with PBS (10 ml) and stored at −80°C for future uHO-1.

Laboratory investigation

Fasting glucose, serum urea, creatinine, urinary creatinine, and microalbuminuria all were assayed by immunoturbidimetry for all groups using Cobas C311 analyzer (Roche Diagnostics, Hoffmann-La Roche). HbA1c was estimated using Cobas Integra 400 plus analyzer (Roche Diagnostics). Urine albumin–creatinine ratio (ACR) was measured for patients in all the groups to compensate for variations in urine albumin concentration in spot urine samples. Estimated glomerular filtration rate (eGFR) is estimated from the modification of diet in renal disease formula as follows: eGFR (ml/min/1.73 m^2)=186×[serum creatinine (mg/dl)]−1.154×[age]−0.203×[0.742 if female]×[1.21 if black].

uHO-1 for all patients was estimated by enzyme-linked immunosorbent assay technique according to the manufacturer's instructions (SinoGeneClon Biotech Co., Ltd), with normal range of 0.06–4 ng/ml.

Statistical analysis

The collected data were analyzed using SPSS 20 software. χ^2-Test and independent *t*-test were used to know the association between different groups. The Pearson's correlation coefficient (*r*) was calculated to assess the correlation between uHO-1 and other parameters of the study. Receiver operating characteristic (ROC) curve and area under the curve were constructed to calculate the optimized cutoff

Table 1 Demographic and laboratory data of the patients and control groups

	Microalbuminuric (group I)	Normoalbuminuric (group II)	Control (group III)	*P* value
Age	52.57±9.46	49.93±12.73	55.75±12.76	0.784
Sex (male/female)	17/28	11/9	10/10	0.153
Glucose	214.75±25.56•,▪	182.20±9.95•	84.56±15.42	0.000
Urea	64.79±11.79•,▪	34.54±2.41•	19.34±8.67	0.001
Creatinine	1.96±0.04•,▪	1.02±0.25•	0.67±0.03	0.000
HBA1c	7.95±2.62	7.13±1.56	5.69±1.27	0.437
Cholesterol	220.70±73.24•,▪	213.04±53.87•	143.42±35.21	0.001
Triglycerides	216.64±34.67•,▪	217.44±98.45•	85.96±53.67	0.000
HDL	44.71±9.34	46.24±10.26	46.8±8.45	0.473
LDL	131.57±64.93•	124.18±53.82•	84.2±6.34	0.000
ACR	2680.35±602.56•,▪	297.98±79.65•	27.56±3.04	0.000
eGFR	49.2±26.2•,▪	77.6±26.0•	120±20.4	0.000
uHO-1	4.91±2.1•,▪	7.61±1.8•	0.36±0.02	0.000

ACR, albumin–creatinine ratio; eGFR, estimated glomerular filtration rate; HBA1c, hemoglobinA1c; HDL, high-density lipoprotein; LDL, low-density lipoprotein; uHO-1, urinary heme oxygenase-1. •$P<0.05$ group I compared with control and group II compared with control. ▪$P<0.05$ group I compared with group II.

points for uHO-1 to reach the best compromise in the prediction of DN. *P* value less than 0.05 was considered statistically significant.

Results

The demographic data and laboratory finding of the patient groups and control group (Table 1) show that there was no significant difference between the three groups in age, sex, glycosylated hemoglobin, and HDL.

There was a highly significant elevation ($P<0.001$) in the mean value of urea and cholesterol and very highly significant elevation ($P<0.000$) in the mean value of fasting glucose, creatinine, triglycerides, and ACR in the two groups of patients compared with control group. Among diabetic groups, there was a very high significant elevation in microalbuminuric group compared with the normoalbuminuric group.

eGFR was highly significantly ($P<0.000$) decrease in microalbuminuric group compared with the normoalbuminuric group and control group, correspondingly, and highly significantly decreased in normoalbuminuric group compared with control group.

uHO-1 was very highly significantly elevated ($P<0.000$) in microalbuminuric group compared with normoalbuminuric group and control group correspondingly and was very highly significantly elevated in normoalbuminuric group compared with the control group.

The correlation between uHO-1 and eGFR and ACR is presented in Table 2. There was a positive correlation between uHO-1 and ACR, with highly statistical significance, and no correlation between ACR and eGFR.

Table 2 Correlations between heme oxygenase-1, estimated glomerular filtration rate, and albumin–creatinine in diabetic patients (*N*=65)

	R	*P* value
ACR	0.374	0.002
eGFR	−0.186	0.137

ACR, albumin–creatinine ratio; eGFR, estimated glomerular filtration rate.

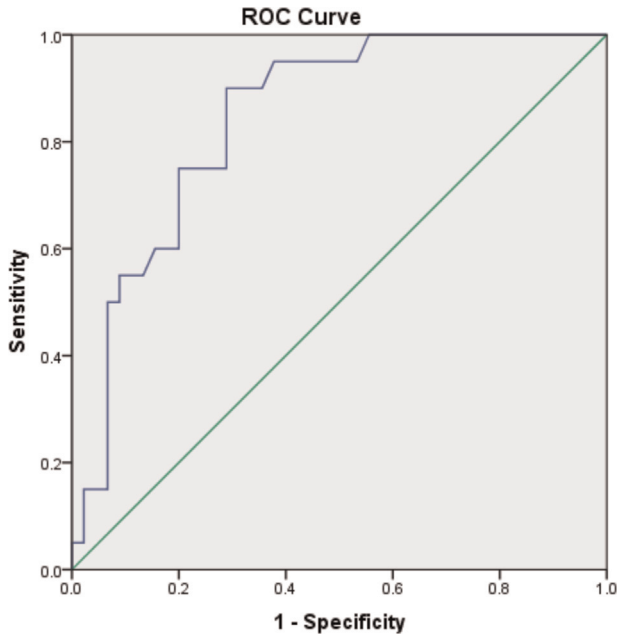
To evaluate the sensitivity and specificity of uHO-1 as a new and early marker for diagnosis of DN, ROC curve was constructed (Fig. 1). It showed a good diagnostic profile, with area under the curve of 0.844 with *P* value less than 0.000 and 95% confidence interval of 0.750–0.939. At a cutoff value of 5.8 ng/ml, the sensitivity was 80% and specificity was 71%.

Discussion

Microalbuminuria has been considered the earliest marker for DN. However, up to 61% of diabetic patients have decrease in renal function before the appearance of microalbuminuria [12]. Albumin can be found in urine in some cases even in nondiabetic ones, such as in infection, after exercise, and with cardiac lesion, and it arises among smokers too, indicating that albumin lacks the specificity for accurate diagnosis of DN [13].

In this study, eGFR was significantly lower in normoalbuminuric group compared with the control group, which means the normoalbuminuric group had decreased renal function. This means microalbuminuria is not the early and ideal marker

Figure 1



Receiver operating characteristic (ROC) curve and area under the curve of urinary heme oxygenase-1 in diabetic patients.

for detection of the decrease in renal function among diabetic patients. Robles-Osorio and Sabath [12], showed that 61.6% patients with GFR less than 60 ml/min were normoalbuminuric. According to the United Kingdom Prospective Diabetes Study, the percentage of patients with decreased renal function without albuminuria was 11% [14]. The study National Health and Nutrition Examination Survey III reports that 13% of type 2 diabetes mellitus patients had GFR less than 60 ml/min, and 36% of them had no albuminuria [15]. So, diabetic patients with normoalbuminuria are in need for a marker to early diagnose impending DN.

Many recent studies showed that renal tubular injury has an important role in the pathogenesis of DN, and this tubular injury may precede the glomerular injury and not secondary to glomerular damage. Therefore, detection of serum or urinary markers of renal tubular injury may help in the early diagnosis of DN such as urinary and plasma levels of neutrophil gelatinase-associated lipocalin and kidney injury molecule-1, which are considered early markers of DN [16,17].

In this study, we estimate the urinary level of HO-1 in diabetic patients and in control group, and we showed that uHO-1 is significantly elevated in microalbuminuric patients compared to normoalbuminuric patients and control group. Most importantly, uHO-1 was high in normoalbuminuric diabetic patients compared with control group. uHO-1 was positively correlated with ACR and that was statistically significant. This means that uHO-1 can be used as a new and early diagnostic marker for DN in diabetic patients.

In this study, ROC curve was constructed to calculate the optimized cutoff points for uHO-1 to reach the best compromise in the prediction of DN than using microalbuminuria which is the gold standard for the diagnosis of DN. Moreover, we assumed a cutoff point of uHO-1 for early prediction of DN. The results may suggest that uHO-1 may be useful in detecting early changes in kidney in diabetic patients.

High glucose levels and diabetic substrates, such as glycation end products, affect renal tubular cells producing tubular cell hypertrophy and the interstitial deposition of chemokines, cytokines, and adhesion molecules, which lead to inflammation and fibrosis of the tubules [18]. The proximal tubule is more affected to these metabolic and hemodynamic factors. Brito *et al.* [19] have shown that the proximal tubular basement membrane is already thickened in normoalbuminuric patients with diabetes.

HO-1 protein is induced in tubular epithelial cells, more prominently in distal tubules rather than proximal tubules, but is not expressed in glomerular cells. HO-1 is expressed in proximal tubules and not the distal one in response to oxidative stress produced by glycation end products, proteinuria, and hematuria. The upregulation of HO-1 protein in renal tubules but not in glomerular cells in diseased kidney may relate to the differential sensitivity and response to oxidant stress exhibited by these cells [8].Bao *et al.* [20] found slightly increased plasma HO-1 concentrations in patients with newly diagnosed type II diabetes (2.4 ng/ml), compared with controls (1.1 ng/ml), which is caused by oxidative effect of hyperglycemia in diabetic. Saukkonen *et al.* [21], reported modest HO-1 increases in pelleted urinary tubular and inflammatory cells obtained from patients with inflammatory renal disease. Li *et al.* [22], showed that uHO-1 was significantly higher in normoalbuminuric compared with control group and significantly correlated with ACR ratio, which is in agreement with our study.

There are some limitations of this study like the small number of patients, the measurement of one marker of tubular injury, and lack of follow-up of the patients.

Conclusion

These findings suggest that uHO-1 can be considered a novel renal biomarker associated with increased

albuminuria and ACR in diabetic patients. The study has also demonstrated that uHO-1 levels increased before the onset of microalbuminuria, which makes it an early biomarker of renal tubular injury and early diagnostic marker of DN in normoalbuminuric patients.

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Conflicts of interest
There are no conflicts of interest.

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